1987

Role of the Pseudomonas Aeruginosa reca Analogue in Lysogeny and Recombination

Tyler Alan. Kokjohn
Loyola University Chicago

ROLE OF THE *PSEUDOMONAS AERUGINOSA RECA* ANALOGUE IN LYSOGENY AND RECOMBINATION

by

Tyler Alan Kokjohn

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

1987

ACKNOWLEDGEMENTS

This dissertation represents a collaborative effort between myself and several other individuals whom I wish to thank for their efforts. The committee assigned to critically evaluate this work consisted of Dr. Stephen Farrand, Dr. Allen Frankfater, Dr. Robert Malone, Dr. Philip Matsumura, and Dr. Robert Miller. The dissertation resulting from their suggestions and labor is superior to that which otherwise would have been produced.

I have benefitted from the presence of several fellow students and investigators at Loyola University. I thank Margo Cavenagh, Pamela Derstine, Frank Mondello, Sue O'Morchoe, Dennis Saye, Sue Simonson and Debbie Tonetti for more things than I can mention. Diane Pischl was, and will continue to be, a standard of excellence.

Phil Matsumura is due special recognition for starting me off on the right foot just as he has done so unselfishly for many others.

Bob Miller contributed an enormous amount of effort to this project. As a scientist he has my respect and as a fine person, my admiration.

DEDICATION

As precisely as I can remember, during the summer of 1963 in the course of a conversation with my father I was informed that a person could earn a doctorate in the sciences. More importantly this could be accomplished by describing something really neat, like how a caterpillar becomes a butterfly! I replied that this is what I would do someday and was informed;

"Your story will have to be very, very detailed. You may even have to describe the chemical reactions that cause what you are seeing."

This dissertation is dedicated to my Mom and Dad who have always helped me in every one of my efforts. In fact, it is not possible to separate their work from mine and I must count them as my most valuable collaborators.

VITA

Tyler A. Kokjohn was born on September 3, 1954, in Sioux City, Iowa. His parents are Walter and Ruth Kokjohn. He received his primary and secondary education in the Sioux City Public School system. He received a Bachelor of Science degree in Biology from Morningside College in 1976. He was employed as a science teacher in the Sioux City School system, teaching eighth grade physical science and ninth grade earth science. He was also an adjunct instructor in the state of Iowa Area XII Education Agency teacher education program.

He entered the graduate program of Loyola University of Chicago in August, 1980. In August, 1982, he began research in the laboratory of Dr. Robert V. Miller. He entered the doctoral program in Biochemistry in 1983.

TABLE OF CONTENTS

PAGE

LIST OF TABLES

PAGE

LIST OF FIGURES

PAGE

PAGE

CHAPTER I

GENERAL INTRODUCTION

Elucidation of the Role of the *Escherichia coli recA* Gene

The systematic study of biology has revealed that organisms, whether simple or complex, reorganize and recombine their genetic material. The genetic material has been shown to consist of DNA molecules (131). This ability to allow genetic material to change in a controlled fashion has certain advantages for the organism. If individual organisms within a population are not genetically identical, the process of evolution, and species survival prospects, may be enhanced (131).

The process of recombination in all organisms seems likely to be complex, requiring several enzymatic activities. In order to dissect the process, the first experimentation directed toward an understanding of the

molecular basis of recombination utilized some of the simpler organisms available for study.

In Escherichia coli the product of the recA gene is required for the process of homologous recombination. The recA gene was first identified in 1965 by Clark and Margulies in a search for cells unable to support homologous recombination (26). Survivors of nitrosoguanidine-treated cultures were screened for cells unable to undergo homologous recombination of genetic information after conjugation. In this study, evidence was presented supporting the conclusion that the mutants isolated were truly impaired in genetic recombination ability and not simply unable to receive DNA during the process of conjugation. Cells containing mutations in the recA gene showed a ten-thousand fold reduction in recombinant formation subsequent to Hfr matings. Since the initial discovery of the recA gene, several phenotypic classes of recA mutations have been identified (Table 1).

The protein product of the recA gene was first identified by McEntee, et al., (88) using a specialized transducing phage of lambda. The product of the E. coli recA gene was estimated to have a molecular weight of 43,000. Utilizing recombinant DNA techniques, Sancar and

Table 1: Alleles of *recA*[a].

Allele class	Example	Cell phenotype	Molecular defect
Wild-type	*recA*$^+$	Resistant to UV irradiation Able to express SOS functions and induce resident prophage Proficient for homologous recombination	Wild-type protein
Defective (Def)	*recA1*	Sensitive to UV irradiation Unable to derepress SOS functions or induce resident prophage after UV; no spontaneous induction of prophage Deficient in homologous recombination	Lack of functional RecA protein
Deficient	*recA142*	Sensitive to UV irradiation Unable to derepress SOS functions or induce resident prophage after UV; spontaneous induction of prophage normal Defective in homologous recombination	Altered RecA protein

(Table 1: Continued)

Allele class	Example	Cell phenotype	Molecular defect
Protease constitutive	recA730 recA441	Constitutive expression of SOS functions in absence of DNA damage Proficient in homologous recombination	Hyperactive proteolytic activity
Split Function	recA430	Sensitive to UV irradiation Defective in expression of SOS functions and induction of prophage Proficient in homologous recombination	Reduced proteolytic function

[a]Adapted from Clark (25) and Ossanna, et al. (103).

Rupp cloned the *E. coli K-12 recA* gene (119). They physically mapped the cloned DNA and expressed the protein product in maxicells. Their estimate of the monomer molecular weight of the RecA protein was 42,000. The following year, Sancar, *et al.*, reported the complete DNA nucleotide base sequence of the *recA* gene (120). The gene was revealed to contain an open reading frame of 1059 nucleotides encoding a protein with a predicted molecular weight of 37,842. No explanation for the size discrepancy between the predicted and observed molecular weights of the RecA protein has been offered.

The mechanism of recombination requires the physical breakage and reunion of DNA molecules (93, 131). The *in vitro* biochemical activities of the purified *recA* gene product are consistent with the protein acting in the pairing of DNA molecules and as a catalyst in reactions that logically would occur in the generation of recombinant DNA molecules. The RecA protein promotes homologous pairing of DNA molecules *in vitro*. The renaturation of complementary single strands of DNA is stimulated (147, 149) and RecA promotes formation of joint molecules , referred to as D-loops, from single-stranded or partially single-stranded DNA and duplexed DNA (90, 135). These paired molecules are joined by

noncovalent interactions, no covalent topological linkage between paired molecules is catalyzed by RecA (30). The RecA-stimulated renaturation of complementary single strands of DNA has been shown to be dependent upon ATP hydrolysis by the RecA protein (146). Both double- and single-stranded DNA stimulate DNA-dependent ATP hydrolysis by RecA (147). McEntee, et al., (90) demonstrated a rapid and preferential binding of RecA protein to single-stranded DNA. They postulated a sequential ligand binding scheme with single-stranded DNA bound first, followed by ATP and double-stranded DNA binding.

Single-stranded DNA plays a special role in the process of homologous pairing of DNA molecules (32). The binding of RecA protein to single-stranded regions stimulates the protein to unwind duplex DNA. RecA protein has been shown to promote the homologous pairing of two circular DNA molecules when one had a single-stranded gap (32). The preferential pairing of the gapped molecule and superhelical DNA occurs when both nicked circular and superhelical DNA are present. In the presence of ATP and divalent cations, the pairing of gapped circular and superhelical DNA occurs as effectively as D-loop formation from single-stranded and duplex DNA.

RecA protein promotes homologous pairing of DNA molecules by an ordered reaction in which the protein first polymerizes on single-stranded regions of DNA (91, 135). The complex may be formed in the presence or absence of E. coli single-stranded binding (ssb) protein (135), with one molecule of RecA for each 3.6 nucleotide residues and up to one molecule of ssb protein for each 15 nucleotide residues. Menetski and Kowalczykowski (91) have noted an aspect of the interaction of RecA protein with single-stranded DNA that may be important in the process of recombination catalyzed by the RecA protein. Their experiments have revealed that the affinity of RecA for single-stranded DNA is modulated by ATP hydrolysis with the affinity of the protein for single-stranded DNA decreasing upon hydrolysis of the bound nucleotide. RecA protein may thus continually cycle on and off single-stranded DNA as strand invasion and partner strand exchange occur in recombination.

The RecA protein acts to promote the recombination of DNA molecules by the catalysis of a remarkable set of reactions termed the strand transferase activity by DasGupta, et al., (32). If either DNA molecule has a single-stranded region, the RecA protein will produce joint structures. The RecA protein, unaided by other

proteins, is capable of causing strand displacement and pairing of duplex DNA with a new partner strand (32). It thus functions not only on strand uptake, but also in donation and receipt of a strand, extension of the region transfered and physical transfer of the invading strand. The polarity of the single-stranded DNA transfered is clearly recognized with transfer proceeding in the 3' to 5' direction on the invading single-stranded DNA (24).

The exact mechanism by which RecA protein brings into register homologous regions of DNA is still not understood. Protection experiments indicate that the protein contacts the single-stranded DNA backbone, leaving portions of the bases free from contact with the protein (81). There is an apparent processive search for homology performed by the RecA protein when the homologous region to be be paired is not in close proximity to the single-stranded region (50).

The RecA protein has been shown to possess an activity capable of generating recombinant molecules without the presence of DNA sequence homology in the molecules joined (110). The RecA protein has been shown to have the ability to join, noncovalently, DNA molecules end to end. This activity has been demonstrated for both single- and double-stranded DNA molecules. The experimenters speculate that this <u>in vitro</u> activity of

RecA may be important in DNA repair especially under conditions resulting in double-stranded DNA breaks. However, a complication arises in that the DNA molecules being held by RecA were not ligatable *in vitro* using T4 ligase suggesting that this *in vitro* activity may only be artifactual.

In *E. coli*, two major pathways generate recombinant DNA and require several other gene products in addition to RecA (25). In the wild-type cell, the major share of recombination is done via the RecBC pathway. The activity of this pathway depends upon the presence in the cell of a functional *recBC* gene product encoding a protein with exonuclease V activity (25). Another route does exist however, the RecF pathway. Normally, the RecF system is expressed at a very low level, with 99 per cent of the recombination in the cell using the RecBC pathway. It is unclear what role the *recF* gene product plays in recombination. In *E. coli recBC* mutants, a second mutation indirectly suppressing the recombination deficiency, *sbcB*, enables the RecF pathway to operate. The *sbcB* mutation causes the loss of a nuclease that is hypothesized to normally shunt an intermediate of recombination into the RecBC pathway and out of the RecF pathway (25).

A number of aspects of the physiology of the recA gene product were discovered subsequent to the identification of its protein product. Inouye and Pardee (68) demonstarted the induction of an E. coli protein, later termed protein X, upon treatment of the cells with nalidixic acid. The induction of protein X was abolished by mutations in the recA or lexA genes. Protein X was ultimately identified as being identical to the recA gene product (52,53,88). These same studies demonstrated that a mutation, tif-1, was an allele of recA. The tif mutation is interesting in that it manifests several novel properties. E. coli mutants containing the tif-1 allele allow induction of lambda prophage upon a shift up in temperature (18,19,25). If the cells are not lysogenic, a lethal filamentation is observed (153). These data indicate that in addition to its role in homologous recombination, the recA gene product is clearly required in a diverse set of cellular activites.

Role of the E. coli recA Gene Product in DNA Repair

That the recA gene product plays a role of some sort in the response of E. coli to UV irradiation was first indicated in the original paper describing the isolation of recA mutations by Clark and Margulies (26).

The recA mutants were dramatically more sensitive to UV irradiation than their isogenic parents. As noted later by Clark, a most interesting aspect of the recA mutations is their pleiotropic nature (25). One of the observations concerning expression of the RecA protein was that it was inducible above baseline levels upon exposure of the cell to agents which damage DNA (41,53). It is now clear that the recA gene product controls the response of a network of unlinked genes in E. coli which, upon structural damage to DNA or interference with normal DNA replication, show increases in their levels of expression. This group of coordinately expressed genes has been termed the SOS system (141,153). The existence of this system was demonstrated through the efforts of a number of investigators in several laboratories.

Maintaining the integrity of the genetic information of an organism is so essential that it is not surprising that E. coli has evolved several, independent, systems for repairing damaged DNA some of which do not require RecA. It had long been known that UV irradiation was capable of killing bacteria. Further experimentation indicated that the most effective wavelength was 254 nm and the assumption was made that the DNA of the cell was the likely target of UV (131). Exposure of E. coli cells

to UV irradiation results in the creation of a number of classes of lesions in the chromosomal DNA (55). The cyclobutyl pyrimidine dimer is the photoproduct formed in DNA in the highest yield upon exposure to 254 nm UV irradiation (55,131). (From this point on, these UV-induced lesions will be simply referred to as thymine dimers, with the understanding that this represents a simplification of the actual condition in the cell.) A direct correlation between cell survival and the number of thymine dimers in chromosomal DNA was shown by Wacker (139).

One method of repairing such DNA damage is to directly reverse the chemical change in the DNA molecule. E. coli has a photorepair enzyme which is capable of the splitting of thymine dimers (131,141). This enzyme is functional only in the presence of visible light of 300-500 nm (139) with no reversal occurring in the dark. The mechanism utilized to effect DNA repair by this enzyme is unknown. It is curious that this protein does not absorb light of any wavelength (139). This repair process is accurate, with no associated mutagenesis. The photoreactivation process of E. coli acts only upon thymine dimers (139). This process does not require RecA protein.

A second repair process recognizes lesions in DNA such as thymine dimers and excises them in a process termed cut-and-patch or excision repair. This repair system was first examined by Setlow and Carrier (127) who determined that thymine dimers are removed, unsplit, from the DNA of excision-competent cells. In sensitive strains, the dimers remain unexcised from the DNA and remain photoreactivable. The onset of DNA synthesis, (i. e., cell proliferation and survival), was correlated with the dimer removal. This process is much more complicated, with several gene products acting in concert to effect removal of the damaged DNA. As noted by Hanawalt, et al., a remarkable feature of the excision and resynthesis steps of excision repair is the multiplicity of enzymes with suitable properties for involvement in the process (55). Howard-Flanders, et al., (65) characterized mutations at three separate loci in E. coli (uvrA, uvrB, uvrC) which make the cells sensitive to UV and unable to remove thymine dimers from DNA. This process is possible only if a nondamaged partner strand is available and capable of serving as template for resynthesis of the excised portion. In this process, an incision is introduced into the damaged DNA strand and a fragment including the lesion is excised

(55). The single-stranded gap remaining in the DNA is resynthesised utilizing the undamaged strand as a template. There is heterogeneity in the sizes of the resynthesized patches with the majority being short (20-30 nucleotides), and a minority long (several hundred nucleotides) (55). Long patch repair appears to be inducible upon DNA damage and is dependent upon the Rec^+ phenotype (15,44). This repair process is accurate.

Rupp and Howard-Flanders (116) found that E. coli strains unable to perform excision repair could survive and continue dividing with approximately 50 thymine dimers in their chromosomal DNA. They noted that if the cells also contained a recA mutation as well as the defect in excision repair, the cells became much more sensitive to UV, tolerating only about one thymine dimer per cell. These data were interpreted as indicating that cells have a recombinational method of DNA repair, thus explaining in part the requirement for the recA gene product. This repair process has been termed post-replication recombinational repair. Rupp and Howard-Flanders proposed that $RecA^+$ cells use this recombinational repair mechanism to survive UV damage to DNA which still contains thymine dimers. DNA synthesized subsequent to UV irradiation contains gaps opposite thymine dimers (65). These gaps are generated when

strand elongation is blocked by the presence of an unexcised dimer. DNA synthesis resumes at some point beyond the dimer leaving a gap in the nascent DNA strand. Post replication recombinational repair occurs by sister strand exchanges. The products of the recombination event still contain thymine dimers. However, a gradual distribution of dimers into nascent DNA occurs, resulting in their effective dilution (116). These unexcised dimers clearly remain intact in uvr mutants, with complete elimination requiring several rounds of replication. Post-replication recombinational repair is postulated to allow the cell to sustain DNA damage without any delay in DNA replication. This pathway of DNA repair requires some functions not needed for generalized recombination, since lexA and recF mutations inhibit this process (15). As noted by Rupp and Howard-Flanders (116), this repair process is accurate, since excision-deficient cells produce progeny with no enrichment in auxotrophic clones.

Functional RecA protein is also required for the activity of the fourth repair system known as the SOS system. Defais, et al., proposed that UV damage induces a cell response that has a common aspect with other phenomena also dependent upon the $RecA^+$ and $LexA^+$

phenotype such as prophage lambda induction (37). The SOS hypothesis of Radman (36,37) stipulates that DNA damage generates a signal responsible for the simultaneous derepression of functions which presumably aid in cell survival.

Induction of Prophage Lambda by *E. coli* RecA Protein

The phenomenon of induction to lytic growth of resident prophage lambda by UV irradiation has been known since the work of Weigle and Delbruck in 1951 (145). In fact, about half of the known temperate phages of distinct immunity that lysogenize enteric bacteria are UV- inducible (111). It has also been determined that other treatments result in prophage induction including X- irradiation, starvation for thymine and addition of Mitomycin C to the cell growth medium (111). The study of the biochemical basis of the induction for lambda prophage led to a greater understanding of the mechanism of activation of the genes of the SOS network and the underlying common aspects of SOS and phage induction.

Jacob and Monod (72) proposed that the *cI* gene product of lambda is a repressor which blocks lytic development of prophage and superinfecting homoimmune phages by selective repression of the expression of one

or more phage genes. The lambda cI repressor protein was first isolated by Ptashne (107) and subsequently characterized in detail by him and others. As predicted on the basis of genetic data, the cI gene product was found to bind specifically to lambda DNA and this binding has been demonstrated to be the mechanism of controlling lytic and lysogenic functions of lambda by the selective repression of expression of subsets of lambda phage genes (108).

Ogawa and Tomizawa (100), using mutants of phage lambda that formed abortive lysogens, demonstrated that the induction of prophage lambda requires one or more cellular functions with no need for expression of phage genes. The phage utilized could neither form stable chromosomally-integrated prophage nor replicate independently. This left primarily cells containing newly-synthesized phage repressor protein in their cytoplasm which was translated during the interval that the phage DNA was present. These cells were immune to any subsequent infection by lambda phage by virtue of the presence of this repressor protein presence. The DNA binding (repressor) activity disappeared from the cells after inducing treatments such as UV irradiation (100). This destruction of repressor subsequent to UV treatment

required protein synthesis. Since both lambda induction and destruction of repressor activity could be blocked by mutations in the recA gene, it was hypothesized that RecA played a direct role in this process. It had been previously noted by Brooks and Clark (16) that lysogens of recA strains are unable to support lambda induction subsequent to treatment of the cells with agents normally able to cause lytic growth of the phage. In addition, the presence of the tif-1 mutation in E. coli allowed the induction of lambda prophage upon growth at elevated temperatures in the absence of any inducing treatments (18,151,152,153). The tif-1 mutation was later identified as an allele of recA (18,41). Roberts and Roberts (112) demonstrated that the induction of prophage lambda via UV irradiation or Mitomycin C treatment results in the specific proteolytic breakdown of the cI repressor, suggesting a biochemical mechanism for the process of induction. Their data indicated that the recA gene product may have a role in the process of prophage induction which likely involved a specific cleavage of lambda cI repressor.

Further studies by Roberts, et al., indicated that purified RecA protein was capable of causing the specific cleavage of cI repressor (113,114). This work provided convincing evidence that the recA gene product was

directly involved in the process of induction as they demonstrated that mutations in recA affected induction activity mediated by the RecA protein in a manner that would be predicted by the phenotype of the cell containing the recA mutation.

Induction of the SOS network in E. coli

In $RecA^+$ strains, induction of SOS functions other than prophage excision and vegetative growth is dependent upon a second gene, lexA. Specific alleles of lexA lead to a non-inducible or constitutive phenotype suggesting that the lexA gene product is a repressor of SOS expression (141).

The lexA gene product was identified as a 24,000 dalton protein (84,85). This polypeptide was shown to be specifically cleaved by the RecA protein in a manner analogous to that of the phage lambda cI repressor (84). No such cleavage reaction was demonstrable for the lexA protein product produced in a lexA3- containing mutant.

Gudas and Pardee (54) found that the synthesis of RecA (protein X) could be blocked by certain lexA mutations. RecA synthesis could be induced by growth of lexA (ts) mutants at elevated temperature. They proposed

that the protein product of the lexA gene acted as a repressor for recA. McEntee (88) and Gudas and Mount (52) also proposed a model with recA expression repressed by the protein product of the lexA gene. RecA is autoregulatory in the sense that it modulates the activity of the protein which acts as the repressor of recA gene expression. Both studies noted that specific activation of the RecA protein was required for the expression of SOS functions.

The SOS system is now known to consist of at least 17 unlinked genes whose expression is activated upon exposure of the cell to agents which damage DNA or interfere with DNA replication (141). The SOS response of E. coli includes prophage induction, inhibition of cell division, an enhanced ability to repair DNA damage and an increase in mutagenesis (153). The product of the lexA gene controls the expression of the unlinked genes of the SOS network in a negative fashion by binding at operator sequences in front of the genes (79,85). Comparison of the operators of LexA-controlled genes revealed DNA sequence homology among them with a conserved concensus sequence (85,141).

It has been hypothesized that lambda repressor has evolved sensitivity to RecA-mediated cleavage because release of phage from a cell which had sustained UV

damage would be advantageous to the phage (99,111). Induction of lambda prophage does require that drastic conditions prevail within the cell. The expression of lytic functions does not begin until 90% of the cI repressor protein in the cell is cleaved (6).

The cellular signal responsible for activating the latent proteolytic activity of RecA is uncharacterized to date. Subsequent to DNA damage, the RecA protein is activated to a state in which it promotes the cleavge of LexA and cI repressors, if present. In order to express the proteolytic-promoting activity of RecA the protein must be specifically activated. Overproduction of RecA is not concomitant with an increase in the proteolytic-promoting activity (109). The activation process is reversible in that the level of activated RecA protein decreases with successful repair of the cell's damaged DNA and is thought to involve a conformational change in the protein (141). However, it has not been determined if activated RecA protein is turned over in the cell and replaced by the nonactivated form during the recovery from DNA damage. It is possible to effect the activation of RecA in vitro upon addition of single-stranded DNA and nucleoside triphosphate (114). The reaction rate of cleavage of LexA protein generated in these in vitro

systems is sufficient to account for *in vivo* induction of the SOS network. No other proteins appear to be needed for this process.

The element common to most models for the mechanism of creation of the SOS signaling entity is that of formation of gapped, single-stranded DNA in the cell (99). One of the most detailed models is that proposed by Roberts and Devoret (111). They propose that single-stranded DNA is exposed when a replication fork encounters an unexcised dimer and the DNA helix is unwound without concurrent DNA synthesis. RecA binds to the single-stranded gap, thereby becoming activated to promote repressor cleavage. Such binding may also initiate recombinational repair. RecA is thus activated upon entering a DNA gap. They propose that the proteolytic activity is inactivated if and when the protein engages duplex DNA in a search for DNA sequences homologous to the bound single-stranded DNA. The concentration of activated RecA and overall rate of repressor inactivation therefore depends on the linear density of DNA lesions. Treatments that abort DNA replication but do not actually damage DNA cause activation of RecA by stalling replication forks thus forming single-stranded gaps. Mutations such as *tif-1 (recA441)* may cause RecA protein to have a greater

affinity for small gaps that presumably occur normally in the DNA and thus to become more easily activated (111, S. Kowalczykowski, personal communication).

The experiments of Kenyon and Walker clarified the nature of the SOS response utilizing the ability of defective Mu phage, Mu d1(Ap^r, *lac*), to fuse in random fashion to genes in the *E. coli* chromosome (79). These phage transpose randomly in cells containing wild-type Mu as helper phage and are constructed to form transcriptional fusions of the *lac* operon to the target gene. These investigators were able to isolate several fusions that demonstrated increased expression upon exposure of the cell to SOS inducing agents. The expression of this set of unlinked, damage-inducible (*din*) genes proved to be dependent upon the *lexA*$^+$ and *recA*$^+$ genotype. Some of the *din* genes have been identified as genes previously known to participate in DNA repair, while others have no known phenotype.

As suggested above, a result of the SOS response is the induction of enhanced repair capacity and mutagenic activity of the damaged cell. The first observation of of this phenomenon was made by Weigle (143). If UV-damaged lambda phage are utilized to infect *E. coli*, host cell repair functions act to repair the phage and thus to

reactivate their ability to produce plaques. The host cell may also mutagenize the infecting phage. This reactivation and mutagenesis is stimulated (induced) by exposure of the E. coli host cell to small amounts of UV irradiation. The dependence of Weigle mutagenesis and reactivation upon the recA$^+$ lexA$^+$ genotype was shown by Defais, et al. (37).

Further investigation of DNA damage-inducible mutagenesis in E. coli has revealed that two additional genes are involved, umuD and umuC (141). These genes are in an operon under control of the LexA protein. In addition, it is possible that UmuC protein requires proteolytic processing in order to become activated to perform its role in mutagenesis and Weigle reactivation (141), since cells deficient in both LexA protein and RecA protein [lexA(def) recA] are unable to support mutagenesis (85). The DNA nucleotide base sequence of umuC suggests possibility that a RecA cleavage site is present (141).

Conservation of the recA Gene

The recA gene appears to have been conserved among bacteria. Utilizing interspecific complementation, recA analogues from several different genera have been

demonstrated to be expressed and to function in E. coli (11,49,78,101,125). An especially interesting aspect of this conservation is that a number of isolates of recA analogues are able to support induction to lytic growth of prophage lambda in E. coli (78). While some of the recA analogue protein products have been shown to be larger than the E. coli protein, analysis of several isolates has revealed a conservation of protein structure (11,78,125).

Rec$^-$ mutants of Pseudomonas aeruginosa

Several recombinationally-deficient mutants of Pseudomonas aeruginosa have been isolated and characterized. Holloway in 1966 (60) isolated several Rec$^-$ mutants of P. aeruginosa PAO. Using the assumption that recombinational mutants would not allow lysogeny, he was able to isolate several mutants subsequent to nitrosoguanidine mutagenesis that were unable to allow establishment of lysogeny and were recombinationally deficient. While the isolation of these Rec$^-$ mutants was based upon a false assumption, the strains were clearly less proficient in the processes of transduction and conjugation than their isogenic parents. Following the

lead of Clark and Margulies (26), Holloway tested the UV irradiation sensitivity of the mutants. Only one strain showed a greater sensitivity than the parental strain. Using nitrosoguanidine mutagenesis and screening for the loss of the ability to support lysogeny, van de Putte and Holloway (136) were able to isolate a temperature-sensitive Rec$^-$ mutant of P. aeruginosa PAO. This strain was recombinationally impaired, unable to allow establishment of lysogeny upon infection by a temperate phage, and unable to form colonies at elevated temperature. They also demonstrated that rare lysogens of phages D3 and F116 constructed by infecting the mutant at a high multiplicity of infection (MOI) were subsequently uninduced by UV irradiation in contrast to wild-type cells. This temperature-sensitive Rec$^-$ strain was no more sensitive to UV irradiation than its parent. It is unfortunate that these strains have been lost and are no longer available for comparison and experimentation.

Chandler and Krishnapillai (23) also isolated Rec$^-$ mutants of P. aeruginosa with similar properties to those described above. These mutants were not mapped and may in fact represent multiple mutations affecting recombination (D. Haas, personal communication).

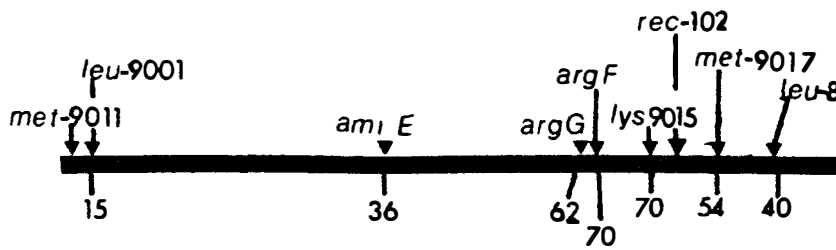
Using a similar approach to isolate P. aeruginosa mutants deficient in the establishment of lysogeny, Miller and Ku in 1978 described several new mutations in lysogeny establishment functions (96). Two phenotypic classes of lysogeny establishment deficient (Les^-) mutants were isolated and characterized. These mutants were unable to be lysogenized except at high moi. The mutations were shown to map in two regions of the P. aeruginosa chromosome. One class, exemplified by lesB908, has pleiotropic effects upon recombinational ability of the cell containing it. Such cells were markedly deficient in the ability to undergo recombination subsequent to transduction or conjugation and were later shown to be more sensitive to UV and X-ray irradiation than the parental strain (96). Cells containing lesA mutations are unable to support lysogeny establishment but are recombinationally proficient. Früh, et al., (43) using nitrosoguanidine mutagenized DNA from strain PAT, reported the construction of several Rec^- strains of P. aeruginosa PAO by screening directly for recombinational deficiency. These strains were found to be deficient in the ability to recombine DNA received by transduction and conjugation. The growth of these strains was inhibited by mitomycin C. While the data is

difficult to interpret, these isolates were apparently more sensitive to UV irradiation than their parents and in comparison to other Rec$^+$ *P. aeruginosa* strains as well. The *rec-102* marker was mapped to the 45 min region of the *P. aeruginosa* PAO chromosome (Figure 1). The authors noted that repeated attempts to lysogenize strains containing the *rec-102* allele with phage F116 failed, but did not give any details of how this construction was attempted.

Utilizing a gene replacement technique, Ohman, *et al.*, constructed *recA* mutants of *P. aeruginosa* strain FRD (101). A transposon-inactivated *recA* gene was homogenotized into the chromosome and the resultant strain characterized. These mutants were extremely sensitive to UV irradiation and showed an impairment in recombinational ability, although limitations in the method used to gauge the deficiency in recombination make it impossible to make meaningful comparisons to wild-type strains.

The *recA* gene of *E. coli* has been well characterized and clearly is multifunctional. Its loss leads to pleiotropic effects in the cell. Studies of the process of lysogeny in *P. aeruginosa* have yielded mutants incapable of supporting the establishment of lysogeny. Some of these mutants are also Rec$^-$ with several

Figure 1. Conjugational linkage map of the P. aeruginosa PAO chromosome. Numbers under the line represent percent linkage of the rec-102 marker to other markers in the 45 min region of the PAO chromosome obtained in R68.45 matings. Abbreviations are as follows: ami; acetamide utilization, arg; arginine biosynthesis, leu; leucine biosynthesis, lys; lysine biosynthesis, met; methionine biosynthesis, rec; recombination function. Adapted from Früh, et al. (43).


rec-102
leu-9001
argF
met-9017
leu-8
met-9011
ami E
argG
lys 9015
15
36
62
70
70
54
40

properties analogous to recA mutants of E. coli. It seems possible that the alteration of a recA analogue function in P. aeruginosa may explain the basis of the pleiotropic phenotype of these Les$^-$ strains. In this study, the recA analogue of P. aeruginosa was isolated and analysis performed to determine its role in lysogeny establishment and recombination in P. aeruginosa.

CHAPTER II

ISOLATION OF THE P. AERUGINOSA RECA ANALOGUE

Mutations in the E. coli recA gene were first isolated by Clark and Margulies (26). The recA gene product is clearly required for the processes of DNA repair (55), homologous recombination (135,146,147), and induction of the network of SOS genes (141). The recA protein promotes the invasion of duplex DNA regions by single-stranded homologous DNA in vitro leading to a D-loop structure (89). When complexed with DNA, the protein exhibits an ATPase activity (147). DNA damage generates an as yet uncharacterized signal which reversibly activates recA protein to induce the SOS network (141). The activated recA protein cleaves the lexA repressor causing induction of the SOS genes (79,85). Under these conditions, phage lambda repressor is also proteolytically cleaved (111) causing the induction to lytic growth of any resident lambda

prophage. While induction effects are thought to involve a direct protease activity of the recA protein itself, the product of the recA gene may in fact be playing an indirect role, perhaps by stimulating an autocatalyic protease activity inherent in the repressors themselves (83). In any case, for induction of prophage or the SOS response, the protein must be activated. The overproduction of RecA protein is not sufficient to cause SOS induction (109).

A number of mutants of P. aeruginosa have been isolated that are recombinationally deficient (23,44,61,96,101). Several of these mutant strains have been demonstrated to be unable to support the establishment of lysogeny upon infection by temperate phages (44,61,96). The lysogeny establishment deficient (Les^-) mutant strains described by Miller and Ku (96) are the best characterized of P. aeruginosa strains exhibiting Les^- and/or Rec^- attributes. Some of the Les^- mutant alleles described by Miller and Ku have characteristics that suggest they may be analogous to some recA mutations of E. coli. P. aeruginosa strains containing the lesB908 mutation are much more sensitive to UV and X-ray irradiation. The cells are also less able perform host cell reactivation of UV irradiated

phage DNA. Cells containing lesB908 are completely deficient in the process of homologous recombination for DNA received by either conjugational or transductional processes. These P. aeruginosa mutants also have a lengthened generation time with a decrease in viability. It seemed possible that at least a subset of Les$^-$ mutants might be caused by mutation of a gene with functions analogous to the recA gene product of E. coli. To investigate this possibility, an attempt was made to clone and characterize the recA analogue of P. aeruginosa.

The technique of interspecific complementation has been utilized to obtain DNA clones which contain the recA genes of several diverse bacterial species (11,41,49,78,94,101). These heterologous genes are expressed in E. coli providing a ready method of isolation of recA analogues from other bacterial genera via functional complementation of defined E. coli recA mutations. This technique was used to isolate a recA analogue of P. aeruginosa PAO.

Materials and Methods

Bacteria and bacteriophage.

The bacterial strains used are listed in Table 2. Bacteriophage are listed in Table 3.

Plasmids.

Plasmid pBR322 (13) was used as the vehicle for isolation of the recA-complementing clones. Plasmid pJC859 was the generous gift of A.J. Clark. It contains the E. coli K-12 recA gene on a 3.3 kilobase pair (kbp) fragment inserted into the BamHI site of pBR322.

Media.

E. coli and P. aeruginosa were grown in Luria broth (LB) (33). Luria broth consisted of 1% tryptone, 1% NaCl and 0.5% yeast extract. Antibiotics were used for selection at the following concentrations: ampicillin, 50 ug/ml; kanamycin, 50 ug/ml; and mitomycin C, 5 ug/ml. Methyl methane sulfonate (MMS), at a final concentration of 0.01% in L-agar (LB containing 1.5% agar), was used for the initial screening of the recombinant library of the P. aeruginosa chromosome in E. coli strain HB101. Pseudomonas minimal medium (96) with 1.5% agar was used for conjugation. Pseudomonas minimal medium (PMM) contained (w/v) 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.05% sodium citrate, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% $(NH_4)_2SO_4$ and 0.4%

Table 2. Bacterial strains.

Strain	Plasmid	Relevant genotype[a]												Other markers	Prophage[a]	Source or reference
		rec	arg	his	hsd	ilv	leu	met	pro	pur	sup	thr	thi			
E. coli																
AB1157		+	E3	-4	+	+	B6	+	A2	+	E44	-1	-1			(26)
HB101		A13	+	+	S20	+	-	+	A2	+	E44	+	-1			(13)
JC158		+	+	+	+	+	+	+	+	+	+	+	-1	*serA6 HfrH*		(26)
JC2926		A13	E3	-4	+	+	B6	+	A2	+	E44	+	-1			A. J. Clark
JC11372		A56	E3	-4	+	+	B6	+	A2	+	E44	-1	-1	*srl310::Tn10*		A. J. Clark
JC13551		del(306)	E3	-4	+	+	+	+	+	+	+	+	-1	*spr51 sfiB103*		A. J. Clark
JC14773	pJC859	A56	E3	-4	+	+	B6	+	A2	+	E44	-1	-1			A. J. Clark
RM1086	pKM2	A56	E3	-4	+	+	B6	+	A2	+	E44	-1	-1	*srl310::Tn10*		JC11372
RM1088	pKML2	del(306)	E3	-4	+	+	+	+	+	+	+	+	-1	*spr51 sfiB103*		JC13551
RM1121		A56	E3	-4	+	+	B6	+	A2	+	E44	-1	-1	*srl310::Tn10*	(λR)	JC11372
RM2306	pKML2	A13	+	+	S20	+	-	+	A2	+	E44	+	-1			HB101
RM2307		+	E3	-4	+	+	B6	+	A2	+	E44	-1	-1		(λR)	AB1157

(Table 2: Continued)

Strain	Plasmid	Relevant genotype[a]												Other markers	Prophage[a]	Source or reference
		rec	arg	his	hsd	ilv	leu	met	pro	pur	sup	thr	thi			
RM2308	pJC859	A56	E3	-4	+	+	B6	+	A2	+	E44	-1	-1		(λR)	JC14773
RM2309	pKML2	A56	E3	-4	+	+	B6	+	A2	+	E44	-1	-1		(λR)	RM1086
RM5010	pBR322	A13	E3	-4	+	+	B6	+	A2	+	E44	+	-1			JC2926
P. aeruginosa																
PAO1		+	+	+	+	+	+	+	+	+	+	+	+			(96)

[a]Genotype symbols follow the conventions recommended by Demerec et al. (38). Abbreviations are as specified by Bachmann (3). Prophage are symbolized by including the name of the phage in parenthesis when present.

Table 3: Bacteriophage lambda strains.

Strain	Relevant genotype	Source or reference
λR	R5am	R. Malone
λmms813	vir	M. Casadaban
λimm^{434}	imm^{434}	H. Echols
λP	P80 cI857 bio-10	F. Stahl

glucose. Amino acids were added at 50 ug/ml of minimal medium. Lysates of bacteriophage lambda were prepared using tryptone agar (1% tryptone, 1% NaCl, 1.5% agar and 0.2 ug thiamine/ml). Lambda top agar was tryptone agar except the agar concentration was reduced to 0.65%. TM buffer (10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$) was used as phage diluent. TE buffer (10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA) was used in the preparation and storage of DNA.

Preparation of bacteriophage stocks.

Plating cultures for bacteriophage lambda were grown as follows. An overnight culture of cells was diluted 1:200 in tryptone broth, (1% tryptone, 1% NaCl, 0.2 ug thiamine/ ml of medium), and grown for 6 h at 37°C with aereation. The cells were harvested by centrifugation at 5,000 x g for 5 min. The cell pellet was suspended in an equal volume of TMN [10 mM Tris (pH 7.4), 10 mM $MgSO_4$, 15 mM NaCl] and incubated with shaking at 37°C for 60 min. These cells were stored at 4°C until use. They retained their plating ability for several days. Phage stocks for experimentation were grown from inocula from fresh plaques. Several dilutions of phage lysates were mixed with 0.1 ml plating bacteria and poured on fresh tryptone agar plates using a soft agar overlay technique (2). The infected cells were incubated 4-8 h at 37°C until lysis was observed. Phage from

plates showing just confluent lysis were collected by scraping the top agar and delivering into 5ml TM containing 10% (v/v) chloroform. Debris was removed by centrifugation at 5,000 x g for 5 min and the phage stock poured off and stored at 4°C until use.

Cloning and DNA techniques.

P. aeruginosa chromosomal DNA was isolated by a modification of the method of Marmur (87). Cultures of fifty ml of cells were grown in LB to early log phase, harvested by centrifugation at 5,000 x g for 5 min, and frozen at -20°C until needed. The cells were suspended in 10 ml of a solution of 150 mM NaCl and 50 mM EDTA (pH 8). Lysozyme was added to a final concentration of 1 mg/ml and the cells incubated at 37°C until lysis was noted by an increase in viscosity of the mixture. At this point, 2 ml of 20% (w/v) sodium dodecyl sulfate (SDS) in TE buffer was added and the mixture was held at 60°C for 10 min. Sodium perchlorate (5M) was added to a final concentration of 1M. An equal volume of a mixture of chloroform and isoamyl alcohol [24:1 (v/v)] was added and the solution shaken 15 min at room temperature. The extract was centrifuged at 12,000 x g for 5 min. The upper (aqueous) layer was harvested and the DNA precipitated by the addition of two volumes of ethanol.

The precipitate was collected by centrifugation and suspended in 1 ml TE. This solution was repeatedly extracted with equal volumes of 24:1 (v/v) chloroform:iosamyl alcohol until a clear interface was obtained. The DNA in the aqueous phase was then precipitated by the addition of 3M potassium acetate to a final concentration of 300 mM and 2 volumes of ice cold ethanol. The precipitate was recovered by centrifugation and suspended in TE.

Restriction endonucleases were purchased from Boehringer-Mannheim Biochemicals, Indianapolis, IN., and used according to the recommended conditions. Low salt buffer was 10 mM Tris (pH 7.5) and 10 mM $MgCl_2$. Medium salt buffer was 10 mM Tris (pH 7.5) 10 mM $MgCl_2$, 50 mM NaCl. High salt buffer was 50 mM Tris (pH 7.5), 10 mM $MgCl_2$ and 100 mM NaCl. Bam buffer was 10 mM Tris (pH 8), 5 mM $MgCl_2$ and 100 mM NaCl. Eco buffer was 100 mM Tris (pH 7.5), 10 mM $MgCl_2$ and 50 mM NaCl. Digestions were allowed to proceed for 60 min at 37°C.

Chromosomal DNA was partially digested with Sau3AI for 60 min at 37°C under standard conditions. The enzyme was titrated by addition of serial two-fold dilutions of the enzyme to reaction mixtures containing a fixed amount of DNA. The reaction was monitored by electrophoresis of the digested DNA on 0.7% agarose gels to estimate the

size of the digested DNA. The relative mixture of DNA and enzyme yielding the maximal amount of digested fragments in the desired 5 kb size range was used to scale up the digestion procedure. A total of 50 ug of DNA was digested, ethanol precipitated, and suspended in a total volume of 200 ul of TE. The DNA was heated to 68°C for 10 min and layered onto a 10-40% (w/v) sucrose gradient prepared in 1 M NaCl, 5 mM Tris-HCl (pH 8), and 1 mM EDTA (107). The gradient was centrifuged in a Beckman SW41 rotor at 25,000 rpm for 17 h. The gradient was fractionated and fractions analyzed for DNA content and size by electrophoresis on a 0.7% agarose gel. Those fractions containing fragments of approximately 5 kbp were pooled and dialyzed against 4 l of TE buffer at 4°C for 16 h.

Plasmid pBR322 was linearized using BamHI as described above and dephosphorylated with calf intestinal alkaline phosphatase following the procedure of Maniatis, et al. (86). DNA to be dephosphorylated was extracted with phenol and chloroform, followed by an extraction with water-saturated ether and ethanol precipitation (86). The DNA was suspended in 50 ul of 50 mM Tris (pH 9), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 1 mM spermidine. Calf intestinal phosphatase was purchased from Boehringer-

Mannheim Biochemicals and used at a concentration of 0.01 units for each microgram pBR322 DNA to be dephosphorylated (86). The enzyme was added to initiate the reaction and digestion was allowed to proceed for 30 min at 37°C. At this time a second aliquot of enzyme was added and the DNA digested for an additional 30 min. The reaction was stopped by addition of 10 ul of 100 mM Tris (pH 8), 1 M NaCl; 10 mM EDTA; 40 ul water; and 5 ul 10% (w/v) SDS. This mixture was incubated at 68°C for 15 min. The DNA was extracted twice with a 1:1 (v/v) phenol/chloroform solution followed by two extractions with water-saturated ether. The DNA was ethanol precipitated. The precipitate was recovered by centrifugation in a Eppendorf microfuge and the pellet washed with 70% ethanol. The dephosphorylated vector pBR322 and insert DNA were suspended at a concentration of 10 ug/ml each in ligation buffer (105). Ligation buffer was 66 mM Tris (pH 7.4), 33 mM NaCl, and 10 mM $MgCl_2$. ATP was added to a final concentration of 0.5 mM and 0.1 Weiss unit of T4 ligase (Collaborative Research, Lexington, MA) was added to start the reaction. Ligation was carried out for 2 h at 16°C. The ligated DNA was used to transform *E. coli* strain HB101 using a calcium chloride technique to generate competent cells (33). Cells were grown to mid-log in LB at 37°C. The cells

were harvested by centrifugation at 5,000 x g for 5 min. The pellet was suspended in one half the original volume of 50 mM $CaCl_2$, incubated at 0°C for 30 min, and harvested by centrifugation as above. The resultant pellet was suspended in one tenth the original volume of ice cold 50 mM $CaCl_2$. After a further 30 min incubation at 0°C, an aliquot of DNA was added to 0.1 ml of cells and incubation continued for 15 min more. The cells were heat shocked by a 2 min incubation at 37°C. One milliliter of LB was added and the cells were incubated for 30 to 60 min at 37°C to allow expression of the plasmid encoded genes. Cells were mixed with lambda top agar and plated on selective medium.

Plasmid DNA for further characterization was isolated from transformants by a cleared-lysate technique (7). Cells containing the plasmid were grown with appropriate antibiotic selection in 250 ml LB at 37°C with agitation. The cells were harvested by centrifugation at 7,000 x g for 5 min using a SS-34 rotor in a Sorvall RC-5B centrifuge. The pellet was suspended in 5 ml of a solution of 20% (w/v) sucrose, 50 mM Tris (pH 8) and 25 mM EDTA. To begin lysis, 10 mg of lysozyme and one ml of 250 mM EDTA (pH 8) were added. The cells were incubated on ice 30 min. To complete lysis, 4 ml of solution of

0.4% Triton X-100, 50 mM Tris (pH 8) and 25 mM EDTA was added very slowly with continous stirring and the cells were incubated at room temperature for approximately 10 min, until a partial clearing and increase in viscosity of the solution was noted. The lysed cells were immediately centrifuged at 20,000 x g for 15 min in a SS-34 rotor. Cesium chloride was added in the amount of 1 g/ ml of lysed cells and ethidium bromide (5mg/ml) was added in the amount of 0.1 ml/ml of lysed cells. This solution was centrifuged in a TL100.2 rotor at 100,000 rpm 16 h in a Beckman TL-100 ultracentrifuge. The resultant gradient was examined under UV light to visualize chromosomal and plasmid DNA bands. Plasmid DNA was recovered with a syringe and the ethidium bromide removed by extracting with isopropanol saturated with 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7). The plasmid DNA was diluted one to four with 50 mM Tris (pH 8), a one tenth volume of 3 M potassium acetate was added, and this was followed by two volumes of ethanol. This solution was held at -20^{o}C overnight to precipitate the DNA. The DNA was recovered by centrifugation at 10,000 x g in the HB-4 rotor for 45 min at -20^{o}C. The pellet was dried and suspended in TE buffer.

Rapid clone analysis.

Cells containing plasmid DNA were analysed

utilizing a rapid clone analysis technique of Maniatis, et al. (86). Clones to be examined were grown overnight at 37°C. A sample of 1.5 ml was centrifuged in an Eppendorf Microfuge and the cell pellet was suspended in 100 ul of ice-cold solution of 50 mM glucose, 10 mM EDTA and 25 mM Tris (pH 8) containing 4 mg lysozyme per ml. The solution was incubated 5 min at room temperature. At the end of the incubation, 200 ul of a solution of 0.2 N NaOH and 1% (w/v) SDS was added and the tube gently inverted to mix the contents. The resultant solution was incubated on ice for 5 min. A solution of potassium acetate was prepared as follows: 11.5 ml of glacial acetic acid was added to 60 ml of 5 M potassium acetate yielding a final solution which was 3 M with respect to potassium and 5 M with respect to acetate. A 150 ul portion of this acetate solution was added ice cold to the lysed cells and the tube vortexed. The resultant solution was held on ice for 5 min. The mixture, now containing a flocculent precipitate, was centrifuged 5 min in an Eppendorf Microfuge. The supernatant fluid was transferred to a fresh tube and an equal volume of a 1:1 phenol:chloroform solution was added. The mixture was vortexed and centrifuged in an Eppendorf Microfuge briefly to separate the phases. The

aqueous layer was removed to a fresh tube and two volumes of ethanol was added. The tube was incubated briefly at room temperature, and centrifuged in an Eppendorf Microfuge 5 min. The resulting pellet was retained, washed with ethanol and dried. The DNA was suspended in 50 ul TE buffer.

Agarose gel electrophoresis.

Agarose (LE) was purchased from FMC corporation, Rockland, ME. Gels were 0.7% agarose in TBE buffer. TBE buffer was 89 mM Tris-borate, 2 mM EDTA, pH 8.1. DNA samples were mixed with an equal volume of tracking dye solution containing 30% (v/v) glycerol, 7% (w/v) SDS, 0.07% bromphenol blue, and applied to the gel. Usually, 50 ng of DNA was applied per slot. Electrophoresis was carried out in TBE buffer at 5 V/cm for a period of 2-3 h. DNA bands were visualized by soaking the gel in a solution containing ethidium bromide at 0.5 ug/ml for 10-20 min and examining the gel under 260 nm light. Completed gels were photographed for a permanent record. Sizes of DNA molecules or fragments were estimated by running standards of known size in parallel on the same gel.

UV sensitivity.

Cells were grown to approximately 1 x 10^8 colony

forming units (CFU)/ml in LB or LB plus 50 ug/ml ampicillin and harvested by centrifugation at 5,000 x g in a Sorvall centrifuge. The cells were suspended in an equal volume of 0.85% saline. Aliquots of 1 ml were placed in 10 cm Petri dishes and exposed to various fluences of UV light using a General Electric germicidal lamp. UV fluence was determined by the use of a UVX radiometer (Ultra-Violet Products San Gabriel, CA). Survivors were determined by plating appropriate dilutions in duplicate of cells on L-agar and incubating overnight at 37^{o}C. When appropriate, the L-agar was supplemented with antibiotic to select for the presence of the plasmid tested. All manipulations subsequent to UV irradiation were carried out under amber light (Kodak Wratten OC) and incubation was done in the dark to prevent photoreactivation.

Bacterial conjugations.

Cells to be mated were grown in LB to approximately 5 x 10^{8} CFU/ml at 37^{o}C in a shaking water bath. The cells were mixed in a ratio of 1 donor:10 recipients, and allowed to conjugate for 2 h at 37^{o}C without shaking. Selection for transconjugants was by acquisition of amino acid prototrophies. Contraselection for donor cells was by amino acid auxotrophy.

Prophage induction.

Lysogens of appropriate strains were isolated by cross streaking cells against a streak of high titer lambda R5am phage on a L-agar. Survivors that were still sensitive to lambda vir and lambda imm^{434} were tested further for the ability to induce the resident prophage. Lysogens were grown to early log phase (20-25 Klett$_{660}$ units), washed, and resuspended in LB with or without mitomycin C at a concentration of 5 ug/ml. The cells were incubated for 150 min in the dark and lysed by addition of one tenth volume of chloroform. Cell debris was removed by centrifugation (5,000 x g, 10 min) and the lysates were titered for phage on BBL tryptone plates (2) using E. coli C600 grown in tryptone broth supplemented with maltose (0.1%, final concentration) as an indicator host.

Efficiency of Plating of Fec$^-$ lambda phage.

E. coli strain C600 to be used for titration of lambda phage was grown in maltose supplemented tryptone broth to approximately 1 x 10^8 CFU/ml. The cells were harvested by centrifugation, suspended in TM buffer, and starved at 37^{o}C for 60 min. Aliquots of cells (0.1 ml) were then mixed with various dilutions of phage and infection was allowed to proceed at room temperature for 20 min. Lambda top agar (2.5 ml) was then added and the

cells plated on BBL tryptone plates. The concentration of plaque forming units (PFU) was quantitated the next day.

Results

Isolation of the P. aeruginosa recA gene.

A genomic library of P. aeruginosa was constructed by cloning a size-fractionated Sau3AI partial digest of chromosomal DNA from strain PAO1 into the BamHI site of pBR322. Chromosomal DNA of approximately 5 kb was utilized for the cloning. This DNA was transformed into E. coli strain HB101 made competent for transformation and transformants were selected on LB ampicillin plates. The library consists of approximately 5,000 independent clones and has a greater than 99% probability of representing the entire P. aeruginosa genome (86,105).

recA mutants of E. coli are unable to grow in media containing methyl methane sulfonate (11). To isolate clones containing the P. aeruginosa recA analogue, the library was screened for clones which restored the MMS resistance of HB101 by plating cells onto L-agar supplemented with 0.01% MMS. Approximately 10^7 cells were inoculated on each plate. After overnight incubation at $37^{o}C$, MMS resistant colonies appeared at a

frequency of approximately $5x10^{-6}$. Control plates with cells containing no plasmid showed no resistant cells. Five clones were tested for the presence of recombinant plasmids. All were found to contain the same sized insert in pBR322. DNA from one of these clones was used to re-transform strain HB101 and the resulting tranformants were screened for MMS resistance. All new transformants were found to be MMS resistant. These clones were tested for restoration of UV resistance as described above (Figure 2). Plasmid DNA from one of the clones was isolated and used for physical characterization and construction of other strains for further experimentation. This plasmid was designated pKML2. A second plasmid construction containing recA analogue activity was isolated from a BamHI library of P. aeruginosa PAO1 DNA constructed independently of the pKML2-containing library . This plasmid was considerably larger than pKML2, containing approximately 20 kb of insert DNA. This plasmid was designated pKML1.

Restriction mapping.

Plasmid pKML2 was physically mapped using several restriction endonucleases (Figure 3). The cloned fragment is approximately 9.2 kb in size and contains two HindIII, one ClaI, one EcoRI, one XhoI, and two BamHI internal

Figure 2. Restoration by pKML2 of resistance to killing by UV irradiation. Cells were grown to a density of approximately 1 X 10^8/ml in LB, pelleted, resuspended in 0.85% saline, and exposed to various fluences of UV radiation. After appropriate dilution in saline, the irradiated cells were plated in duplicate on L-agar and incubated at 37°C over night in the dark. (○) HB101 (recA13); (●) RM2306 (recA13, pKML2). Mean values are plotted. Range of data is indicated by bars.

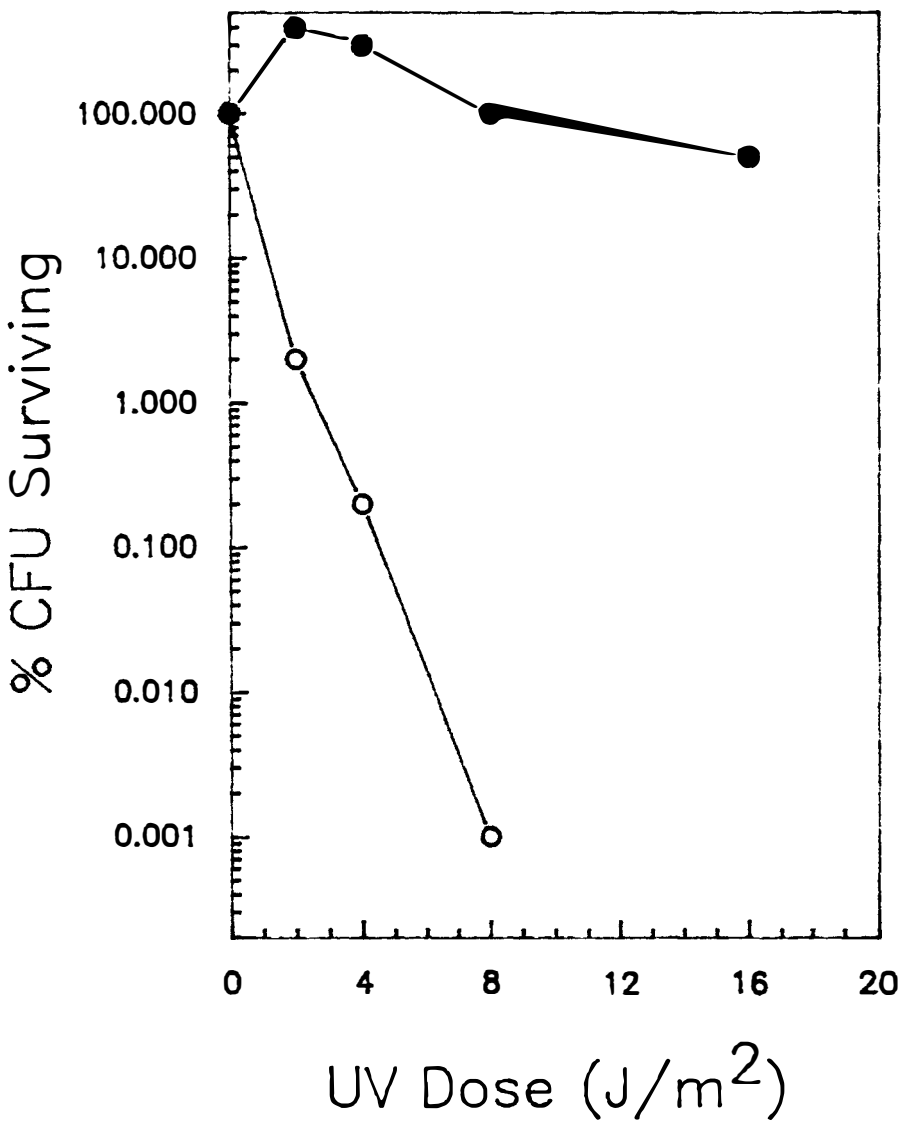
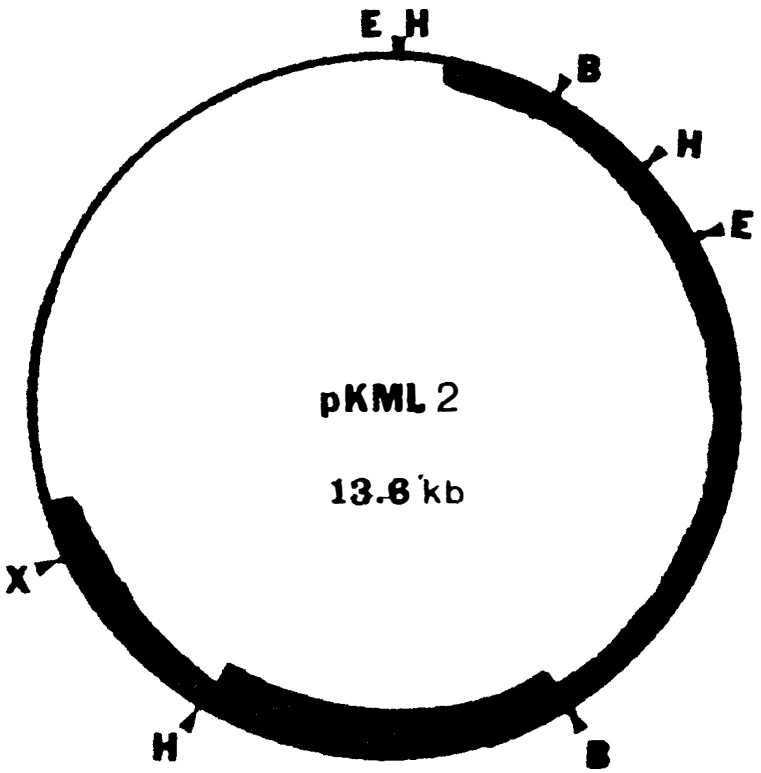

100.000
10.000
1.000
0.100
0.010
0.001
% CFU Surviving
0
4
8
12
16
20
UV Dose (J/m2)

Figure 3. Restriction endonuclease map of pKML2. The thin line represents the vector pBR322; the thick line represents *P. aeruginosa* chromosomal DNA. B = *BamHI*; E = *EcoRI*; H = *HindIII*; X = *XhoI*. Size is expressed in kilobase pairs.


E H
B
H
E
pKML 2
13.6 kb
X
H
B

restriction sites. At the present level of refinement, the restriction map of the P. aeruginosa recA gene and the restriction maps of the recA genes of several enteric species (78,101) do not appear to be related.

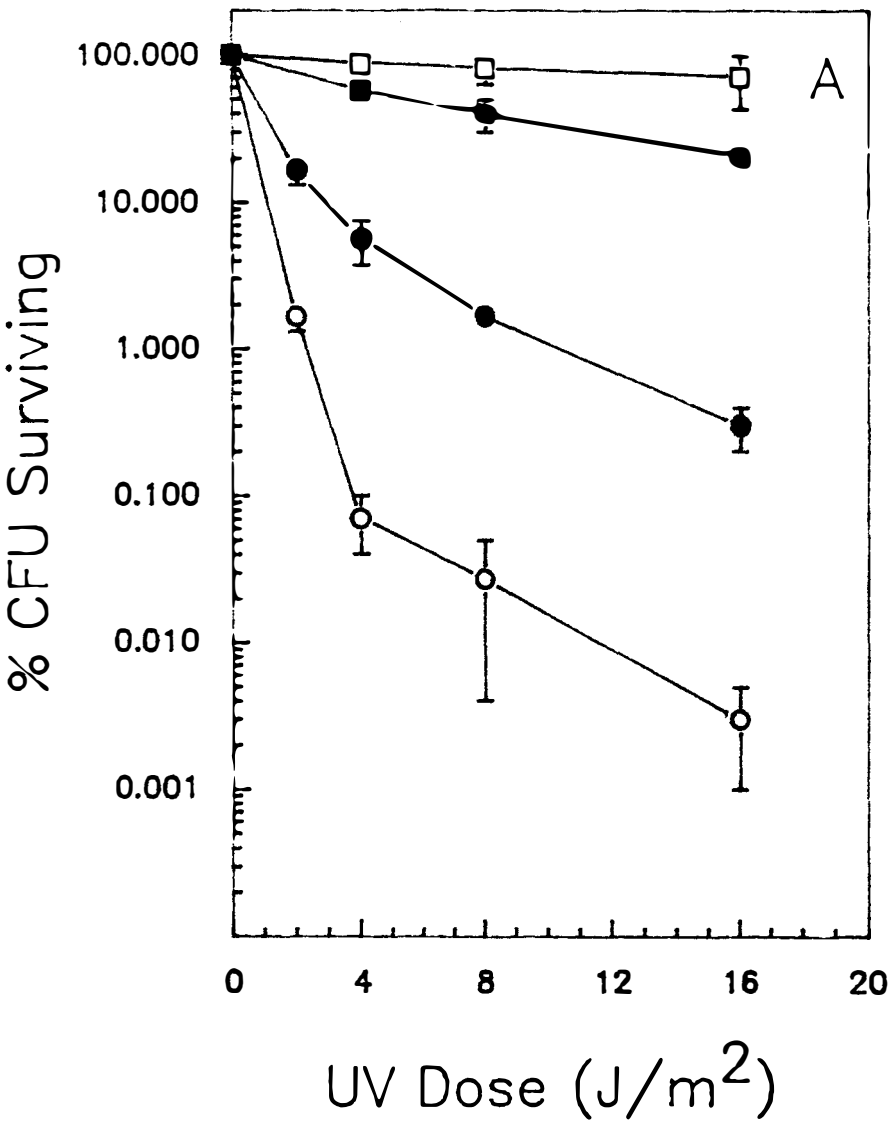
Determination of UV sensitivity.

As discovered initially by Clark and Margulies (26), recA mutations of E. coli have a pleiotropic effect upon the UV resistance of cells containing them. Such cells are much more sensitive than Rec^+ cells. The ability of pKML2 to complement the UV sensitivity of several standard recA mutations of E. coli K-12 was determined. pKML2 DNA was isolated and transformed into these strains, and UV survival was determined as described in Materials and Methods. pKML2 was found to restore the UV resistance of strains containing either a deletion, del(srl-recA)$_{306}$::Tn10 (Figure 4B), or a point mutation, recA56, (Figure 4A) of the recA gene.

Recombinational proficiency.

E. coli cells containing recA mutations are essentially completely deficient in carrying out the process of homologous recombination (25). The ability of pKML2 to restore the ability of various recA recipients to carry out homologous recombination after conjugation was determined (Table 4). Recombinational proficiency was restored in the presence of pKML2. It must be noted

Figure 4. Restoration of resistance to killing by UV irradiation of various RecA$^-$ mutants of E. coli K-12 by pKML2 or pJC859. Experiments were carried out as described for Figure 1. Mean values are shown. A: (□) AB1157 (Rec$^+$); (○) JC11372 (recA56); (■) JC14773; (recA56, pJC859); (●) RM1086 (recA56, pKML2).


100.000
10.000
1.000
0.100
0.010
0.001
% CFU Surviving
A
0
4
8
12
16
20
UV Dose (J/m2)

(Figure 4, continued)

B: (□) AB1157 (Rec$^+$); (o) JC13551 (<u>recA</u> del306); (●) RM1088; (<u>recA</u> del306, pKML2); (▵) RM5010 (<u>recA13</u>, pBR322).

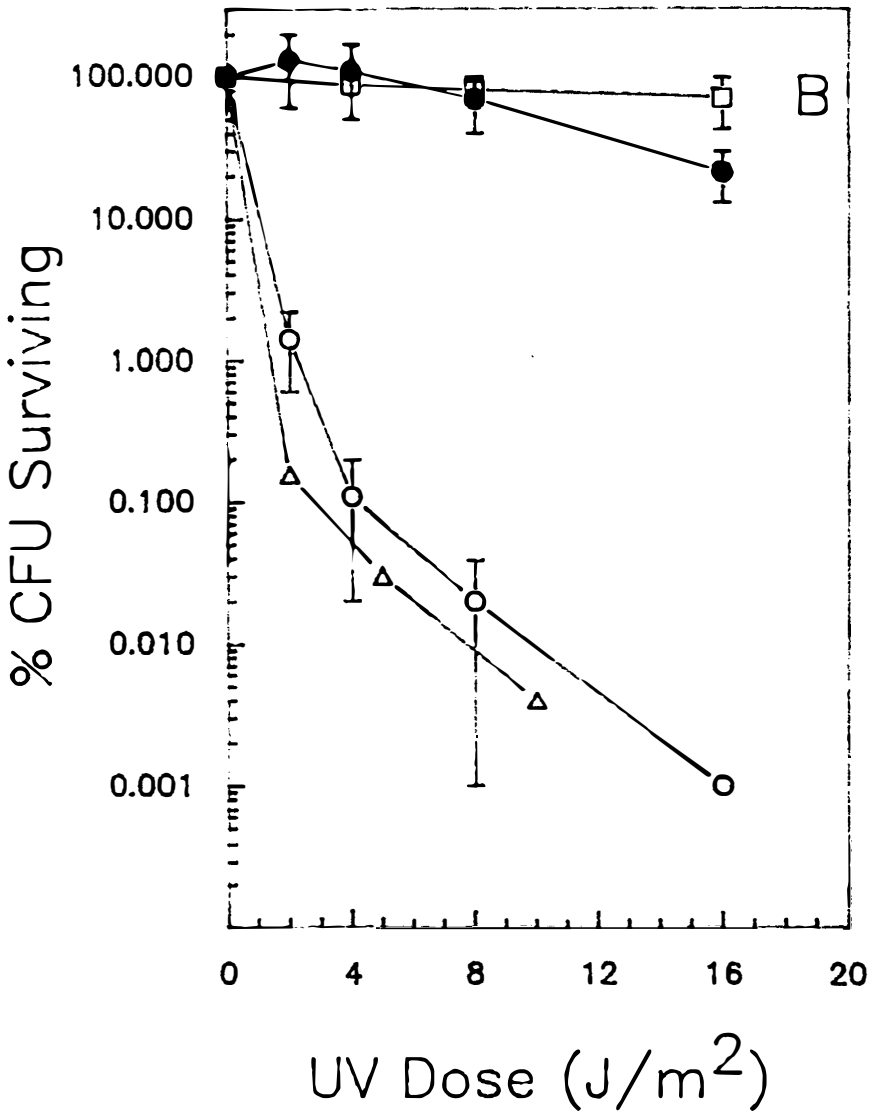

100.000
10.000
1.000
0.100
0.010
0.001
% CFU Surviving
B
0
4
8
12
16
20
UV Dose (J/m2)

Table 4. Recombinational proficiency in Hfr crosses[a]

Strain	Relevant Characteristics	Expt[b]	Recombinational Proficiency[c]
AB1157	Rec$^+$	1	4.3
		2	0.5
JC11372	recA56	1	0.0007
		2	0.00005
JC14773	recA56; pJC859[d]	1	4.3
		2	0.75
RM1086	recA56; pKML2[b]	1	1.0
		2	0.1
HB101	recA13	1	<0.004
RM2306	recA13; pKML2[d]	1	0.4

[a]JC158 was used as the donor strain. Cells were mixed in a ratio of 1 donor:10 recipients. Matings were performed for 2 h at 37^{o}C. Contraselection of the donor was by serine auxotrophy.

[b]Recombinational proficiency of AB1157 strains was determined in two separate experiments. Data from both experiments are shown. HB101 strains were tested once.

[c]proA$^+$ recombinants/100 donors. Data not corrected for possible F' formation.

[d]pJC859 contains the E. coli recA gene. pKML2 contains the P. aeruginosa recA analogue.

that some of the prototrophic clones resulting from the conjugation may actually represent F' elements that exist independently of the chromosome and thus do not represent true recombinants. However, the total contribution of such repliconation in relation to true recombination is insignificant when the comparison is made between isogenic strains.

Suppression of the Fec$^-$ phenotype.

Strains of E. coli containing recA mutations are unable to support the growth of red gam lambda phage such as lambda P80 (25). A lambda phage containing mutations in red and gam genes is dependent upon the host recombination system to generate DNA structures suitable for packaging into mature capsids. Loss of gam gene function prevents normal rolling-circle replication since the host recBC activity, normally antagonized by the gam gene product, is able to digest the linear concatamers generated (25). If such phage are also mutated in red gene function, normally responsible for recombination of phage DNA, production of progeny depends upon RecA-mediated recombination of unit length phage chromosomes into multimeric forms suitable for encapsidation. The efficient maturation of phage thus depends upon a functional host recA gene product. The ability of strains containing pKML2 to plate such phage was

quantitated as described in Materials and Methods. As shown in Table 5, the efficiency of plating (EOP) of Fec$^-$ phage on recA mutants containing pKML2 is essentially the same as the EOP on wild-type E. coli.

Prophage induction.

Resident lambda prophage cannot be induced from RecA$^-$ E. coli cells (16). Normal induction is dependent upon the RecA protein becoming activated resulting in the cleavge of the lambda cI repressor followed by vegtative phage growth. Agents which interfere with normal DNA replication or damage DNA will cause RecA activation (141). The spontaneous and mitomycin C-induced phage production levels from RecA$^-$ lambda lysogens with and without pKML2 was determined. Spontaneous and mitomycin C-stimulated induction in the presence of pKML2 is very dramatically stimulated (Table 6).

Discussion

The product of the recA gene of E. coli has been shown to possess two activities (85,111,141). First, it acts as a synaptic protein in generalized homologous recombination, and second, it can be activated to promote the specific cleavage of the lexA and lambda cI gene

Table 5. Growth of Fec$^-$ lambda phage on *recA* strains with and without pKML2.

Strain	Relevant Characteristics	PFU/ml	EOP[a]
AB1157	Rec$^+$	3.5×10^9	1.00
JC11372	*recA56*	10	3×10^{-9}
JC14773	*recA13*; pJC859[b]	3.7×10^9	1.06
RM1086	*recA13*; pKML2[b]	2.0×10^9	0.57

[a]Relative to the efficiency of plating on the Rec$^+$ strain AB1157. EOPs were determined in one experiment. Phage dilutions were plated in duplicate and the averages are reported.

[b]pJC859 contains the *E. coli recA* gene. pKML2 contains the *P. aeruginosa recA* analogue.

Table 6. Induction of λR prophage by mitomycin C.

Strain	Relevant Characteristics		Viability[a] (CFU/ml)	Bacteriophage Produced (PFU/10^6 CFU)	
				Spontaneous	Induced[b]
RM2307	Rec^+	(1)	2×10^8	2000	10,000,000
RM1121	*recA56*	(1)[c]	6×10^7	1	1
		(2)	6×10^7	1	1.5
RM2309	*recA56*; pKML2[d]	(1)	2×10^8	1,000	20,000
		(2)	3×10^8	670	1,000,000
RM2308	*recA56*; pJC859[d]	(1)	1×10^8	200	10,000
		(2)	1×10^8	100	1,000

[a]At time of induction.

[b]Cells were treated with 5 ug mitomycin C/ml and incubated in the dark at 37°C for 150 min.

[c]Experiments were done in duplicate except where indicated. All phage dilutions were plated in duplicate.

[d]pJC859 contains the *E. coli recA* gene. pKML2 contains the *P. aeruginosa recA* analogue.

products. A DNA fragment derived from the P. aeruginosa PAO chromosome has been isolated and partially characterized which complements both of these activities.

This clone of the P. aeruginosa recA analogue allows functional complementation of defects in homologous recombination in E. coli mutants as judged by the levels of recombinants generated upon Hfr-mediated conjugation and the suppression of the Fec^- phenotype of phage lambda. These data indicate that this clone restores homologous recombination acting upon both closed-circular and linear substrates.

The isolated P. aeruginosa recA-complementary clone restores the ability of the recA host cells to repair UV damage to DNA as judged by increased levels of survival of irradiated cells. This restoration is not as complete in the recA56 background as that conferred by the cloned E. coli recA gene. This may be due to the reduced ability of E. coli RNA polymerase to interact with the P. aeruginosa promoter or the reduced ability of the P. aeruginosa recA protein to interact with the E. coli lexA repressor. Additionally, it is possible that the presence of a full-sized recA56 gene product in the cell containing the recA analogue results in a mixed multimer that is inefficient in the expression of certain RecA activities. RecA probably exists as a tetramer in the

cell (90). *E. coli* cells with a deletion of *recA* are more efficiently complemented by the *P. aeruginosa recA* analogue, suggesting that the presence of mutant RecA in the cell may interfere with expression of RecA functions. It is clear that a strain containing the plasmid pJC859 in the *recA56* background is complemented for RecA functions more efficiently than the same cell containing the *P. aeruginosa* analogue. However, such cells containing pJC859 are not restored to fully wild-type resistance, especially at higher UV fluence. One possible interpretation of these results is that the presence of mutant RecA molecules interferes with the formation of active multimers.

The clone allows both spontaneous and mitomycin C-stimulated induction of lambda prophage from $RecA^-$ *E. coli* mutants at levels approaching those found in $RecA^+$ strains. Before this phenomenon can occur, the *recA* protein must be activated to its proteolytic-promoting state (109,141). The effect of the *recA* analogue upon the spontaneous and UV-stimulated induction of prophages of *P. aeruginosa* will be investigated in the following chapters.

CHAPTER III

CHARACTERIZATION OF THE PSEUDOMONAS AERUGINOSA RECA ANALOGUE

In *E. coli* the protein product of the *recA* gene is required for homologous recombination and initiation of expression of the SOS network following exposure of the cell to DNA damaging agents (25,141). Activated RecA protein also promotes the cleavage of the bacteriophage lambda *c*I repressor causing induction of the lambda prophage to lytic growth (111).

The *E. coli recA* gene product has been identified as a polypeptide of approximately 42,000 daltons by SDS-polyacrylamide gel electrophoresis (119). RecA proteins examined from other bacterial genera have exhibited some differences in size and reactivity to anti-*E. coli recA* antibody (78).

Analogues of the *E. coli recA* gene have been isolated from several diverse species of bacteria by complementation in *trans* of *E. coli recA* mutations

(11,41,49,78,94,101). These heterologous genes have been expressed at least nominally in E. coli and Southern analysis has revealed DNA sequence homology among several isolates (11,78,125). In the previous chapter, the isolation and preliminary characterization of the P. aeruginosa recA analogue was described. In this chapter these analyses are extended, the recA-complementing sequences are more precisely delineated, and the protein product of the recA analogue is identified.

Materials and Methods

Bacteria and bacteriophage.

The bacterial strains used are listed in Table 7. The bacteriophages used are described in Table 8.

Plasmids.

Plasmids pKML1 and pKML2 contain the P. aeruginosa recA analogue within approximately 25 and 9.2 kilobase pair (kb) chromosomal DNA fragments respectively (Chapter II). Plasmids pKML2003 and pKML2004 are subclones of pKML2 that contain the entire P. aeruginosa recA analogue on smaller-sized chromosomal DNA fragments in the vector pBR322. Plasmid pKML2005 is a deletion derivative of pKML2003 with the PvuII fragment removed. Plasmid

Table 7. Bacterial strains.

Strain	Plasmid	Relevant genotype[a]												Other markers	Prophage[a]	Source or reference
		rec	arg	his	hsd	ilv	leu	met	pro	pur	sup	thr	thi			
594		+	+	+	+	+	+	+	+	+	+	+	-1	lac3350 rpsL		(144)
A585		B1009	+	+	+	+	B6	+	+	+	E44	-1	-1	tonA21		F. W. Stahl
AB1157		+	E3	-4	+	+	B6	+	A2	+	E44	-1	-1			(26)
χ1488		+	+	-53	R2	-277	+	C65	+	E41	+	+	-1	minA1 minB2		(27)
GW1031		A56	E3	-4	+	-	B6	+	+	+	+	-1	-1	lac(U169) sulA11 dinB1::Mud(Amp^r, lac)		G. W. Walker
HB101		A13	+	+	S20	+	-	+	A2	+	E44	+	-1			(13)
JC158		+	+	+	+	+	+	+	+	+	+	+	-1	serA6 HfrH		(26)
JC2926		A13	E3	-4	+	+	B6	+	A2	+	E44	-1	-1			A. J. Clark
JC11372		A5	E3	-4	+	+	B6	+	A2	+	E44	-1	-1	srl310::Tn10		A. J. Clark
JC14773	pJC859	A56	E3	-4	+	+	B6	+	A2	+	E44	-1	-1			A. J. Clark
JM103	F'-proAB lacI^{q}Z	+	+	+	+	+	+	+	A2	+	E44	+	-1	lac sbcB		S. Kaplan
RM1086	pKLM2	A56	E3	-4	+	+	B6	+	A2	+	E44	-1	-1	srl310::Tn10		A. J. Clark

(Table 7: Continued)

Strain	Plasmid	Relevant genotype[a]												Other markers	Prophage[a]	Source or reference
		rec	arg	his	hsd	ilv	leu	met	pro	pur	sup	thr	thi			
RM1139	pKML303	A56	E3	-4	+	-	B6	+	+	+	+	-1	-1	lac(U169) sulA11 dinB1::Mud(Amp^r, lac)		GW1031
RM1184		+	E3	-4	+	+	B6	+	A2	+	E44	-1	-1		(λR)	AB1157
RM1185		+	E3	-4	+	+	B6	+	A2	+	E44	-1	-1		(λ 207)	AB1157
RM1186		A56	E3	-4	+	+	B6	+	A2	+	E44	-1	-1	srl310::Tn10	(λ R)	JC11372
RM2310	pKML2003	A13	+	+	S20	+	-	+	A2	+	E44	+	-1			HB101
RM2311	pKML301	A13	+	+	S20	+	-	+	A2	+	E44	+	-1			HB101
RM2312	pKML302	A13	+	+	S20	+	-	+	A2	+	E44	+	-1			HB101
RM2313	pKML303	A13	+	+	S20	+	-	+	A2	+	E44	+	-1			HB101
RM2314	pKML2004	A13	+	+	S20	+	-	+	A2	+	E44	+	-1			HB101
RM2315	pKML2003	A56	E3	-4	+	+	B6	+	A2	+	E44	-1	-1	srl310::Tn10		JC11372
RM2316	pKML2003	A13	E3	-4	+	+	B6	+	A2	+	E44	-1	-1			JC2926
RM2317	pKML2004	A13	E3	-4	+	+	B6	+	A2	+	E44	-1	-1			JC2926
RM2318	pKML2003	A56	E3	-4	+	+	B6	+	A2	+	E44	-1	-1	srl310::Tn10	(λR)	JC11372

(Table 7: Continued)

Strain	Plasmid	Relevant genotype[a]												Other markers	Prophage[a]	Source or reference
		rec	arg	his	hsd	ilv	leu	met	pro	pur	sup	thr	thi			
RM2319	pKML2003	A56	E3	-4	+	+	B6	+	A2	+	E44	-1	-1	srl310::Tn10	(λ207)	JC11372
RM2325	pKML2003	+	+	-53	R2	-277	+	C65	+	E41	+	+	-1	minA1 minB2		X^{1488}
RM2326	pKML2006	+	+	-53	R2	-277	+	C65	+	E41	+	+	-1	minA1 minB2		X^{1488}
RM5000	pKML301	+	E3	-4	+	+	B6	+	A2	+	E44	-1	-1			AB1157
RM5001	pKML2031 F'-proAB $lacI^{q}Z$	+	+	+	+	+	+	+	AB	+	E44	+	-1	lac sbcB		JM103
POII1681		+	F	+	+	+	+	+	AB	+	+	+	-1	lac rpsL	(Mu dII1681)	(20)

[a]Genotype symbols follow the conventions recommended by Demerec et al. (38). Abbreviations are as specified by Bachmann (3). Prophage are symbolized by including the name of the phage in parenthesis when present.

Table 8. Bacteriophage.

Strain	Relevant genotype	Source or reference
Phage Lambda		
λR	*R5am*	R. E. Malone
λmms813	*vir*	F. W. Stahl
λ207	*cI ind*	F. W. Stahl
λ467	*b221 rex::Tn5 O29am P80am*	(35)
Mu Phage		
Mu dII1681	*cts62::IS121* d(Kan^r, *lacZYA*)	(20)

pKML2005 contains the recA complementing activity on an approximately 1.5 kb DNA fragment. Plasmid pKML2006 is a Rec^- deletion derivative of pKML2003 with the segment between BamHI and BglII deleted. Plasmid pKML302 is a Tn5 insertion derivative of pKML2003 with the recA-complementing activity abrogated. Plasmid pKML301 is a Tn5 insertion derivative of pKML2. This plasmid has a Tn5 insertion in the same region and in the same orientation as pKML302, with the concomitant loss of recA-complementing activity. Plasmid pKML303 is a Tn5 insertion derivative of pKML2003. This construction inactivates the ampicillin resistance of the vector, but leaves the recA-complementing activity of the clone intact. Plasmid pKML303 confers a phenotype of ampicillin sensitivity (Amp^s), kanamycin resistance (Kan^r) and recombination proficiency ($RecA^+$).

Plasmid pJC859 contains the E. coli K-12 recA gene inserted into the BamHI site of pBR322 (Chapter II).

Media and chemicals.

Cells were grown in LB at 37°C. Yeast extract maltose (YEM) contained 1% (w/v) tryptone, 0.25% (w/v) NaCl, 0.01% (w/v) yeast extract and 0.2% (w/v) maltose. M9 minimal medium consisted of 0.6% (w/v) Na_2HPO_4, 0.3% (w/v) KH_2PO_4 0.05% (w/v) NaCl, 0.1% (w/v) NH_4Cl, 0.03% (w/v) $MgSO_4(7H_2O)$ and 0.4% (w/v) glucose (86). PMM

(Chapter II) containing 1.3% (w/v) agar, 0.4% (w/v) glucose and supplemented with appropriate amino acids, (25 ug/ml) was used for conjugations. Lysates of bacteriophage lambda were prepared using tryptone agar (Chapter II). Antibiotics were used at the following concentrations : ampicillin, 50 ug/ml; kanamycin, 50ug/ml unless otherwise stated. TM buffer was used as phage diluent and TE buffer was used to store DNA. These buffers were described in Chapter II. Cells used for ultraviolet irradiation sensitivity and conjugation studies were diluted in saline, [0.85% (w/v) NaCl]. Buffered saline glucose (BSG) was 0.85% (w/v) NaCl, 0.03% (w/v)KH_2PO_4, 0.06% (w/v) Na_2HPO_4 and 0.01% (w/v) gelatin (8). Agarose was purchased from FMC corporation (Rockland, ME). Acrylamide and N,N'-methylene-bis-acrylamide were purchased from Bio-Rad laboratories (Richmond, CA). Agar, casamino acids, tryptone, yeast extract and lactose MacConkey agar (129) were purchased from Difco (Detroit, MI). All other chemicals and antibiotics were purchased from Sigma Chemical Co.(St. Louis, MO).

DNA techniques.

Restriction endonuclease digestions were performed as described in Chapter II. Deletion analysis of pKML2

and pKML2003 was performed by restriction digestion followed by re-ligation under conditions favoring intramolecular ligation (105). DNA concentrations of the digest were adjusted to < 5 ug/ml and ligated for 1 h at room temperature using T4 ligase. Deleted plasmids were transformed into E. coli recA mutants using a calcium chloride technique (33) and the RecA phenotype of the transformants examined by testing the ability of the plasmid-containing cells to grow on L-agar supplemented with 0.01% (v/v) MMS or 2 ug Nitrofurantoin (NF)/ml of medium (122).

Tn5 mutagenesis of pKML2003.

Our procedure was similar to that of Bartlett and Matsumura (8). Plasmid pKML2003 was introduced by transformation into the nonsuppressing E. coli strain 594. E. coli 594 (pKML2003) was grown to mid-log phase in YEM at 30°C and Tn5 delivered by infection with lambda 467 (35) at an moi of approximately five. Phage absorption was allowed for 20 min at room temperature and the infected cells were then incubated for 2 h at 30°C.

Cells were plated on L-agar containing 400 ug kanamycin/ml to enrich for Tn5 insertions into the plasmid (67). After incubation for an additional 16-20 h, the plates were scraped and plasmid DNA isolated by a rapid clone analysis technique (86). This plasmid DNA was

utilized to transform E. coli HB101 (33). Selection for transformed clones was made by plating on L-agar containing ampicillin and kanamycin and incubating overnight at 37°C. Transformants were screened for the RecA$^-$ phenotype by testing the ability of cells to grow on L-agar containing MMS or NF as described above. The positions of the Tn5 insertions abolishing recA-complementing activity were mapped using restriction analysis.

Plasmid pKML303 was constructed using the same technique for obtaining Tn5 insertions into the target plasmid. However, selection was made only for kanamycin resistance and survivors were screened for an ampicillin sensitive, RecA$^+$ phenotype.

Southern analysis of the recA analogue.

Plasmids pKML2 and pJC859 were digested with BamHI and electrophoresed on a 0.7% agarose gel as described in Chapter II. The DNA was denatured by soaking the gel in 3 volumes of 1.5 M NaCl and 0.5 M NaOH for 60 min at room temperature with constant stirring. The gel was neutralized by soaking in 3 volumes of a solution of 1 M Tris-HCl (pH 8) and 1.5 M NaCl for 60 min at room temperature with constant stirring. The DNA was blotted onto a nitrocellulose filter (Schleicher and Schuell,

Inc., Keene, NH, BA85 0.45 um pore size) using capillary transfer as described by Maniatis, et al. (86). The transfer buffer was 10X SSC (1.5 M NaCl, 0.15 M Sodium citrate, pH 7.0) SSC buffer (1X) is 150 mM NaCl, 15 mM Sodium citrate, pH 7.0. Transfer was allowed to proceed for approximately 16 h. At the conclusion of transfer, the blot was baked at 80°C, in vacuuo, for 2 h to affix the DNA to the filter. Plasmid pKML2 was labeled using a nick translation kit purchased from Enzo Scientific Co. (New York, NY). The plasmid probe was labeled using the nonradioactive nucleotide Biotin-11-dUTP obtained from BRL (Gaithersburg, MD). Separation of labeled probe from unincorporated nucleotide was accomplished by the spun column procedure of Maniatis, et al. (86). DNA hybridization and detection of bound probe were carried out according to the recommendations of BRL using a modification of the method of Wahl, et al (140). Filters were prehybridized in a solution of 45% formamide, 5X SSC, 5X Denhardt's solution and 250 ug/ml denatured calf thymus DNA. Denhardt's solution was 0.02 % (w/v) polyvinylpyrrolidone, 0.02% (w/v) Ficoll 400 and 0.02% (w/v) Bovine serum albumin (BSA). Prehybridization was done for 1 h at 42°C. The hybridization mix consisted of 4 parts prehybridization solution mixed with 1 part 50 % (w/v) Dextran sulfate (500,000). The probe was denatured

by being heated to 95°C for 5 min followed by quick cooling in an ethanol/ice bath. The probe was added in the amount of 200 ng per ml of hybridization solution. The prehybridization solution was removed as completely as possible from the plastic bag containing the blot and the hybridization solution added. Hybridization was allowed to run 16 h at 42°C. The blot was washed twice in 250 ml of 2X SSC containing 0.1% (w/v) SDS at room temperature for 3 min. This was followed by two washes in 250 ml of 0.2X SSC, 0.1% (w/v) SDS at room temperature for 3 min. The blot was then washed twice in 250 ml of 0.16X SSC, 0.1 % (w/v) SDS for 15 min at 50°C. The blot was rinsed in 2X SSC at room temperature. Bound probe was visualized using reagents provided in the BRL kit and their recommended procedure was followed.

Northern analysis of the recA analogue.

RNA to be examined was isolated from P. aeruginosa PAO1 using a modification of the procedure of Bialkowska-Hobrzanska, et al. (12). All glassware to be used for RNA isolation was baked for 2 h and any plastic containers or apparatus autoclaved. Gloves were worn when manipulating RNA and during all phases of its isolation. Cells were grown to 30 Klett units in LB at 37°C. A 20 ml portion of the culture was mixed with an equal volume of frozen,

crushed 150 mM NaCl, 50 mM EDTA and 50 mM Tris (pH 8). The cells were harvested by centrifugation and frozen at -20°C. The cell pellet was suspended in 250 ul of 10 mM Tris (pH 8), 10 mM EDTA, 100 mM NaCl. An equal volume of this same buffer containing 1% (w/v) SDS was added and the mixture held at 100°C for 5 min. The cell extract was allowed to cool to 60°C. An equal volume of phenol equilibrated with 50 mM Sodium acetate (pH 5.5) was added after warming to 60°C. The mixture was held at 60°C for 5 min. The aqueous phase was retained and the phenol extraction repeated. The resultant aqueous phase was extracted twice with a solution of 24:1 (v/v) chloroform:isoamyl alcohol. The RNA was precipitated by addition of a one tenth volume of 3 M potassium acetate and two volumes cold ethanol. The precipitate was recovered by centrifugation for 5 min in an Eppendorf Microfuge. The pellet was suspended in 200 ul TE, a portion used for spectrophotometric quantitation, and the remainder frozen at -20°C until electrophoretic analysis. The RNA was analysed using 1% agarose containing 2.2 M formaldehyde as described by Perbal (105). The running buffer used was 20 mM MOPS (3-[N-morpholino]propanesulfonic acid [sodium salt]), 5 mM Sodium acetate (pH 7) and 1 mM EDTA. The RNA was denatured prior to loading on the gel using formamide and

formaldehyde as described by Perbal (107). The RNA was mixed with a solution containing a final concentration of 2.2 M formaldehyde, 50% (v/v) formamide, 1X MOPS buffer. The mixture was heated to 60°C for five min, one fourth volume of loading solution was added and the sample immediately electrophoresed. Loading buffer consisted of 50% (v/v) glycerol, 1 mM EDTA, 0.4% (w/v) bromophenol blue and 0.4% (w/v) xylene cyanol. Formaldehyde and formamide were deionized prior to use by stirring with Mixed bed resin [AG 501-X8 (D), Bio-Rad] for 30-60 min. If fresh ACS reagent grade chemicals were used this step was usually not necessary. Approximately 5 ug of RNA was applied per slot and the gel was electrophoresed at 5V/cm for 2-3 h with constant buffer recirculation. RNA within the gel was blotted to nitrocellulose following the procedure of Perbal (105) using capillary transfer. The gels were soaked in 250 ml 20X SSC for 30 min and blotted to nitrocellulose using an apparatus identical to that for Southern transfer. The transfer buffer was 20X SSC and the blotting was allowed to proceed for approximately 16 h. The filter was baked at 80°C <u>in vacuuo</u> for 2 h to affix the RNA. The blot was probed with nick-translated pKML2003 DNA, labeled using α-^{32}P CTP using the same procedure as for the Southern blot. The blot was

prehybridized 20-24 h in a solution of 50% (v/v) formamide, 5X SSC, 5X Denhardt's solution, 0.1% (w/v) SDS and 250 ug denatured calf thymus DNA/ml solution at 42°C. The blot was then placed in hybridization solution containing labeled pKLM2003 probe denatured as described for the Southern blotting. The hybridization solution consisted of 4 parts of the prehybrization solution plus one part 50% (w/v) dextran sulfate. The probe was labeled using conditions to obtain an activity of 10^8 dpm/ug DNA. Hybridization was carried out at 42°C for 24 h. The blot was washed under conditions of high stringency. The blot was washed four times in a solution of 2X SSC containing 0.1% (w/v) SDS for a period of five min at room temperature. This was followed by two washes in a solution of 0.1X SSC containing 0.1% SDS (w/v) for a period of 15 min at 45°C. The blot was air dried and autoradiographed.

Mini-Mu d(Kan^r, lac) insertions into the recA analogue.

Plasmid pKML2003 was introduced by transformation into *E. coli* strain POII1681 (20). This strain contains the Mini-Mu d(Kan^r *lac*) fusion-generating defective prophage Mu dII1681(Kan^r, *lac*) and helper Mu to provide transposition functions. Transposition of the Mu lysogens was initiated by thermal induction. Cells to be

induced were diluted 1:100 in LB plus appropriate antibiotics and grown to mid-log phase at 30°C. The culture was shifted to 42°C and incubated for 20 min. At the conclusion of this high-temperature incubation, the culture was incubated in a 37°C bath with constant agitation until visible lysis of the cells or 2.5 h had elapsed. The lysate was transferred to sterile Eppendorf tubes, 50 ul of chloroform was added, and the tubes were centrifuged to sediment debris. Phage were stored at 4°C with 0.5% (v/v) chloroform. The lysates were used within 5 days since Mu phage are quite unstable. E. coli M8820 (Mu) was utilized as the recipient for transduction. To a fresh overnight culture grown in LB $MgSO_4$ and $CaCl_2$ were added to a final concentration of 10 mM and 5 mM respectively. Phage were absorbed to cells by incubating for 20 min at room temperature with no agitation. Normally, optimal results were obtained using 25 ul of phage and 100 ul of cells. The transduced cells were incubated at 30°C for 90 min to allow expression of plamid and phage drug resistance genes. The cells were plated on LB agar plus ampicillin and kamamycin and incubated overnight at 30°C. The plates were scraped and plasmid DNA prepared by the rapid clone analysis technique described in Chapter II and used to transform

E. coli HB101. Ampicillin and kanamycin resistant cells were screened for the loss of recA-complementing activity by testing for the loss of the ability to grow on medium containing 2 ug NF/ml. The positions and orientations of the Mini-Mu insertions into pKML2003 abolishing recA-complementing activity were determined by restriction endonuclease mapping.

Plasmid DNA was isolated from clones containing Mini-Mu inactivated recA analogue plasmids and used to transform E. coli JM103. The Lac phenotype of E. coli JM103 containing Mini-Mu insertion derivatives of pKML2003 in either orientation was examined by plating cells on lactose MacConkey agar or on agar containing melibiose (2 mg/ml) as the sole carbon source and incubating the plates at 42°C (129). In order for E. coli JM103 to grow at 42° with melibiose as the sole carbon source, LacY must be expressed to allow transport of this sugar into the cell. Other transport mechanisms are inoperative at this temperature. This expression in JM103 is dependent on lacY transcription in the fusion plasmid construction. This melibiose growth test allows the transcription through the fusion construction to be detected by LacY expression even if LacZ is not expressed due to the creation of an out of reading frame fusion.

Minicell analysis of pKML2003.

Plasmids to be analyzed were introduced into the minicell-producing strain E. coli $_X$1488 (27). Minicells were handled as detailed by Clark-Curtiss and Curtiss (27) and Goldberg and Mekelanos (49) with minor modifications. An overnight culture was diluted 1:100 in LB containing appropriate antibiotic and grown approximately 14 h at 37°C. The culture was harvested by centrifugation at 5,000 x g for 5 min. The supernatant fluid was retained and centrifuged at 10,000 x g for 10 min. The resultant minicell pellet was suspended in 1.5 ml BSG. Minicells were purified by centrifugation at 3,500 x g for 15 min through 10-40% (w/v) sucrose gradients. Prior to the sucrose gradient purification step, it was absolutely necessary to vortex the minicells vigorously to remove any attached full-sized cells. The middle portion of the minicell band was recovered from the first gradient using a pipette and run on a second sucrose gradient. The sucrose gradients were prepared by freezing a solution of 22% (w/v) sucrose in BSG and allowing the mixture to thaw at 4°C for 16 h (7).

Minicells were labeled using [^{35}S]-methionine (specific activity 1114 Ci/mmol; New England Nuclear, Boston, MA). Minicells were suspended at an $A_{595}^{1\ cm}$ of 1.0 in M9 minimal medium supplemented with 0.4 % (w/v)

glucose and histidine at 25 ug/ml. [^{35}S]-methionine was added at the rate of 50 uCi/ml and labeling was carried out at 37^{o}C for 45 min. Cold methionine (100 ug/ml) was added and incubation continued for 5 min. Labeled minicells were recovered by centrifugation in an Eppendorf Microfuge. Minicells were suspended in 1/40 volume of TE and an equal volume of sample buffer [125 mM Tris-HCl (pH 6.8), 4% (w/v) sodium dodecyl sulfate, 20% (w/v) glycerol, 10% (v/v) 2-mercaptoethanol] was added. The mixture was boiled for 5 min, 0.1 volume of 0.07 % (w/v) bromphenol blue tracking dye was added and the sample was loaded onto an SDS-polyacrylamide gel for analysis.

SDS polyacrylamide-gel electrophoresis and autoradiography of labeled proteins.

The discontinuous system of Laemlli (80) was used for analysis of proteins, with a stacking gel of 4% (w/v) acrylamide, 2.7% (w/v) bis-acrylamide, 125 mM Tris (pH 6.8), 0.1% (w/v) SDS, 0.5% (w/v) ammonium persulfate, 0.02% (v/v) TEMED and a resolving gel of 12% (w/v) acrylamide, 2.7% bis-acrylamide, 375 mM Tris (pH 8.8), 0.1% (w/v) SDS, 0.5% (w/v) ammonium persulfate, 0.05% (v/v) TEMED. Slab gels (14 x 14 cm) were run at 25 mA, constant current. The electrophoresis buffer was 20 mM

Tris (pH 8.3), 192 mM glycine, 0.1 % (w/v) SDS. Molecular weight standards (BioRad) were run in parallel to allow extrapolation of the molecular mass of the minicell proteins. The molecular mass standards were: lysozyme, M_r 14,400; soybean trypsin inhibitor, M_r 21,500; carbonic anhydrase M_r 31,000; ovalbumin, M_r 45,000; bovine serum albumin, M_r 66,200 and phosphorylase B, M_r 92,500. The gels were stained with a 1% (w/v) solution of Commassie Brilliant Blue for 30-60 min. Gels were destained in a solution of 50% (v/v) methanol, 10% (v/v) acetic acid for 60 min. Destaining was completed in a solution of 7% (v/v) acetic acid, 5% (v/v) methanol until the bands of the size marker proteins were clearly visible. Destained gels were impregnated with sodium salicylate (pH 7) by soaking in a 1M solution for 30 min, dried using a BioRad (Rockville Centre, NY) model 443 slab dryer and autoradiographed at -70°C (22).

Induction of dinB1::Mud(Amp^r, lac) transcription.

Our procedure was similar to that of Kenyon, et al. (79), with minor modifications. Assay technique and solutions were as described by Miller (94). E. coli strains GW1031 and RM1139 were grown in M9 medium containing 1% casamino acids to a density of approximately 2 x 10^8CFU/ml. The cells were harvested by

centrifugation at 5,000 x g for 5 min and suspended in an equal volume of saline. The cells were then exposed to UV irradiation at a fluence of 2 J/m^2 for strain GW1031 or 10 J/m^2 for strain RM1139. The irradiated cells were incubated in the dark and aliquots assayed for β-galactosidase activity at various times after irradiation. β-galactosidase activity was monitored by removing a 1 ml sample of cells and adding 0.5 ml to 0.5 ml assay buffer containing 100 ug chloramphenicol/ml and storing on ice until the time of assay. Assay buffer consisted of 60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM 2-mercaptoethanol. The pH of this solution was adjusted to 7.0. The remainder of the cells was added to 0.5 ml of assay buffer containing 0.5% (v/v) formaldehyde. This formaldehyde-containing tube was used to determine cell density by measuring absorbance at 600 nm. Cells were assayed for activity by adding 40 ul of a 0.1% (w/v) SDS solution in water and 40 ul chloroform, vortexing 10 sec and incubating at room temperature 10 min. This mixture was warmed to 30°C and 40 ul of a 4 mg/ml solution of o-nitrophenyl-B-D-galactoside (ONPG) was added. The reaction was terminated by adding 0.5 ml of 1 M sodium carbonate. The reaction was allowed to proceed for 30 min. Cell debris

was removed by centrifugation in an Eppendorf microfuge and the absorbance of the solution at 420 nm determined. Units of B-galactosidase were calculated as follows (79):

$$\text{Units} = \frac{A_{420} \times 1.5 \times 1000}{A_{600} \times \text{time (min)}}$$

UV Sensitivity of strains containing transposon-inactivated recA analogues.

The presence of multicopy plasmids encoding truncated recA gene products has been shown to cause RecA$^+$ E. coli strains to become sensitive to UV irradiation (126). This effect was postulated to be due to the production of truncated RecA polypeptides upon induction by UV irradiation which interfered with the normal activities of full-sized RecA protein molecules. The production of a radiosensitive phenotype in the cell was correlated inversely with the size of the truncated RecA polypeptide. If the polypeptide was too small, less than 25% of the full-sized RecA protein, no sensitization of the cell occured. This effect was interpreted as being due to interference with the normal formation of RecA protein multimers by the presence of the truncated polypeptides. The mixed multimers obtained were incapble of performing the functions of full-sized multimers.

RecA$^+$ strains of *E. coli* containing translational fusions of *lacZ* to *recA* on multicopy plasmids have been found to become sensitive to UV irradiation (148). This effect was explained as being due to the production of increased amounts of a fusion protein upon induction to high-level expression by UV irradiation which is lethal to the cell.

The effects of the presence of plasmids containing a Tn*5*-inactivated *recA* analogue and *recA* analogue *lacZ* fusions was examined in UV-irradiated RecA$^+$ strains of *E. coli*.

Other methods.

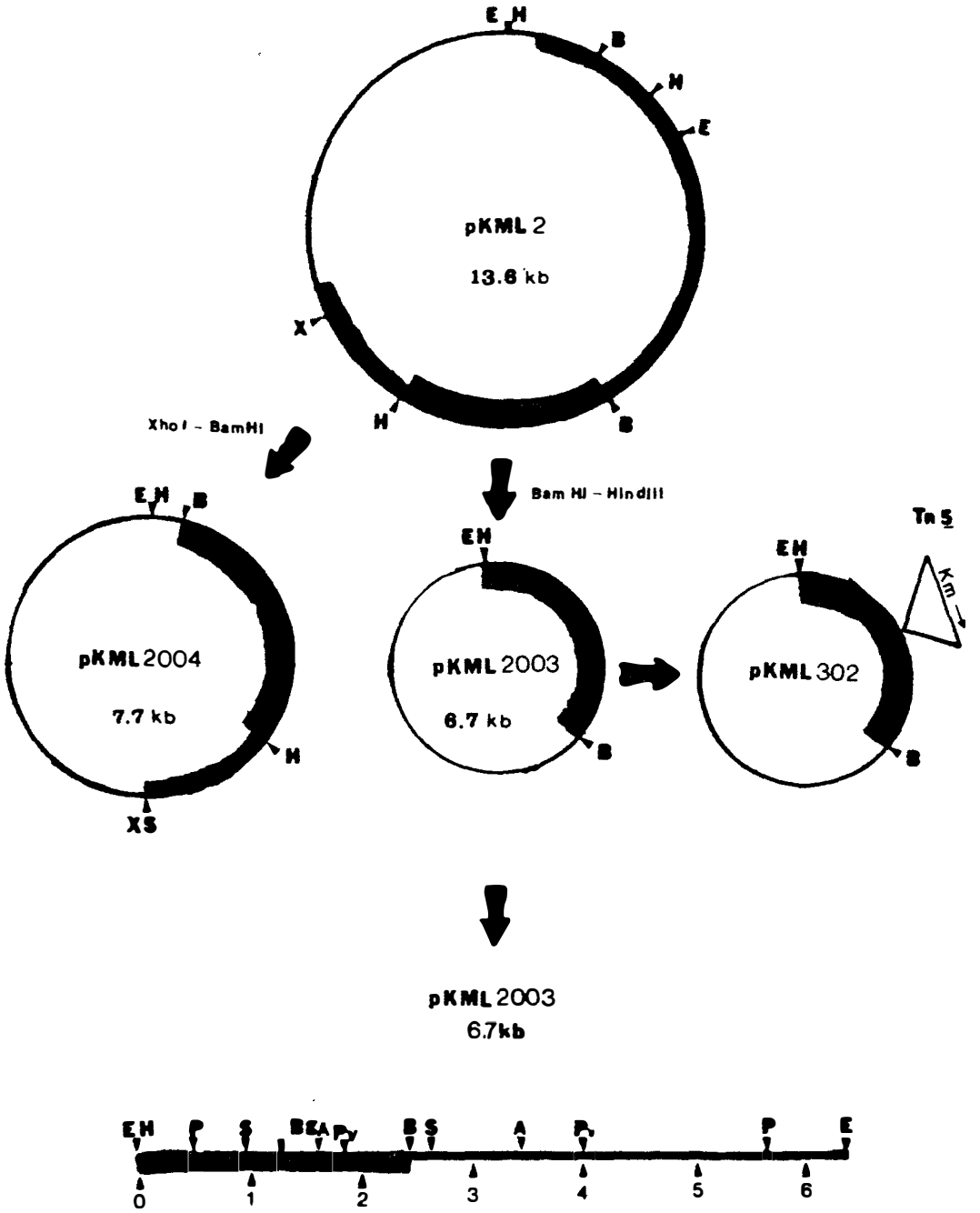
UV sensitivity, bacterial conjugations and phage lambda induction were performed as described in Chapter II.

Results

Restriction mapping and deletion analysis of pKML2 and pKML2003.

Plasmid pKML2 contains the *P. aeruginosa recA* analogue on an approximately 9 kb fragment of DNA cloned into the *Bam*HI site of pBR322 (Figure 5). This DNA fragment is able to complement several *E. coli recA* mutants in *trans*, allowing growth of cells containing the

Figure 5. Restriction endonuclease maps of *P. aeruginosa* *recA*-containing plasmids. The thin lines represent pBR322 DNA; the thick lines represent *P. aeruginosa* chromosomal DNA. The bottom line is a detailed map of pKML2003. Symbols: A, *AvaI*; B, *BamHI*; Bg, *BglII*; E, *EcoRI*, H, *HindIII*; P, *PstI*; Pv, *PvuII*; S, *SalI*; X, *XhoI*. Size is expressed in kilobase pairs. The *recA* complementing activity was contained within the *BamHI*-*HindIII* fragment at 6 o'clock on pKML2.

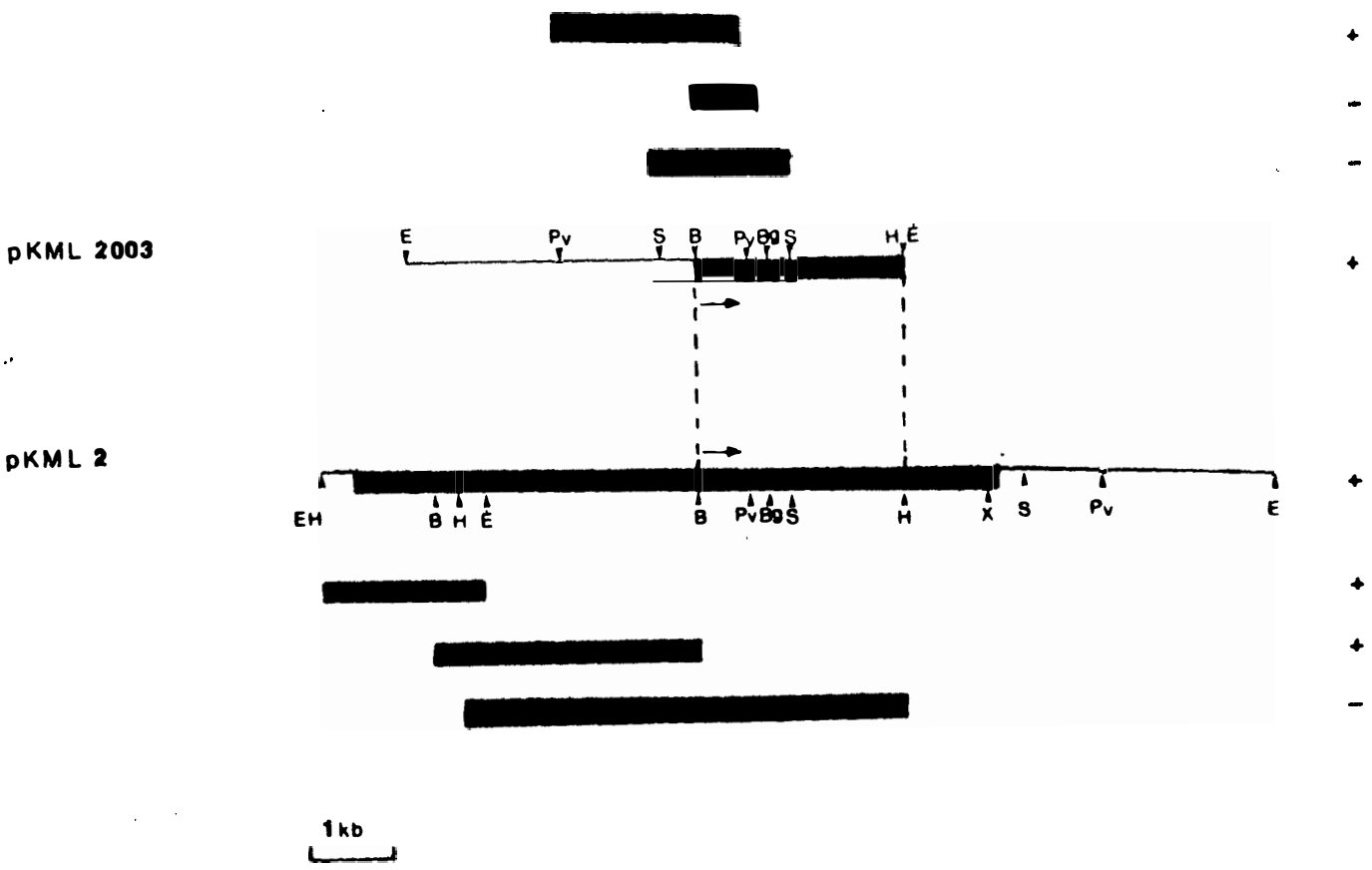

E H
B
H
E
pKML 2
13.6 kb
X
H
B
Xho I - BamHI
Bam HI - HindIII
Tn 5
Km
pKML 2004
7.7 kb
pKML 2003
6.7 kb
pKML 302
XS
pKML 2003
6.7kb
0
1
2
3
4
5
6

cloned DNA in medium containing MMS or NF (Chapter II).

In order to delineate more precisely the location and extent of the analogue coding and control sequences, a number of subclones were constructed and tested for the ability to allow growth of cells in medium containing MMS or NF (Figure 6). Deletion of restriction fragments from pKML2, followed by religation and transformation into E. coli HB101 was utilized to generate several clones. Deletion analysis of pKML2 indicated that the recA-complementing activity is contained within the BamHI-XhoI fragment of the insert DNA (Figure 6). Subclones of this region were generated and examined for the $RecA^+$ phenotype. The 3.5 kb BamHI-XhoI fragment was cloned into pBR322 by replacing the BamHI-SalI fragment of the vector. This clone, pKML2004, is $RecA^+$ and has the insert DNA in the same orientation as pKML2. A subclone containing the 2.3 kb BamHI-HindIII fragment of P. aeruginosa DNA was constructed by replacing the BamHI-HindIII fragment of pBR322 (Figure 5). This subclone, pKML2003, is also $RecA^+$ and is in an orientation opposite to that of pKML2 and pKML2004.

Subcloning experiments have thus revealed that the recA analogue is contained within a 2.3 kb BamHI-HindIII DNA fragment. This fragment was physically mapped (Figure 5 and 6) and subjected to deletion analysis. This

Figure 6. Localization of the *P. aeruginosa recA* analogue. Ability to complement *E. coli recA* mutations was correlated to the physical map to localize the *recA* analogue's position on plasmids pKML2 and pKML2003. Deletions were generated by restriction endonuclease digestion and religation of the deleted plasmid. The region deleted is indicated by the line immediately below and above the maps of pKML2 and pKML2003. The ability of the resultant construction to complement the *E. coli* RecA$^-$ phenotype was tested and is indicated in the column to the right. Arrows indicate direction of transcription of the *recA* analogue (See text and Figure 3 for details). Symbols: B, *BamHI*; Bg, *BglII*; E, *EcoRI*; H, *HindIII*; Pv, *PvuII*; S, *SalI*; X, *XhoI*.


REC
pKML 2003
pKML 2
E
Pv
S
B
Pv
B
B
S
H
É
EH
B
H
É
B
Pv
B
B
S
H
X
S
Pv
E
1 kb

analysis indicated that the recA-complementing activity was contained in the HindIII-PvuII fragment and that the gene extends at least to the BglII site. Restriction analysis of pKML2003 revealed no cleavage sites for HpaI or XbaI.

Tn5 mutagenesis of pKML2003.

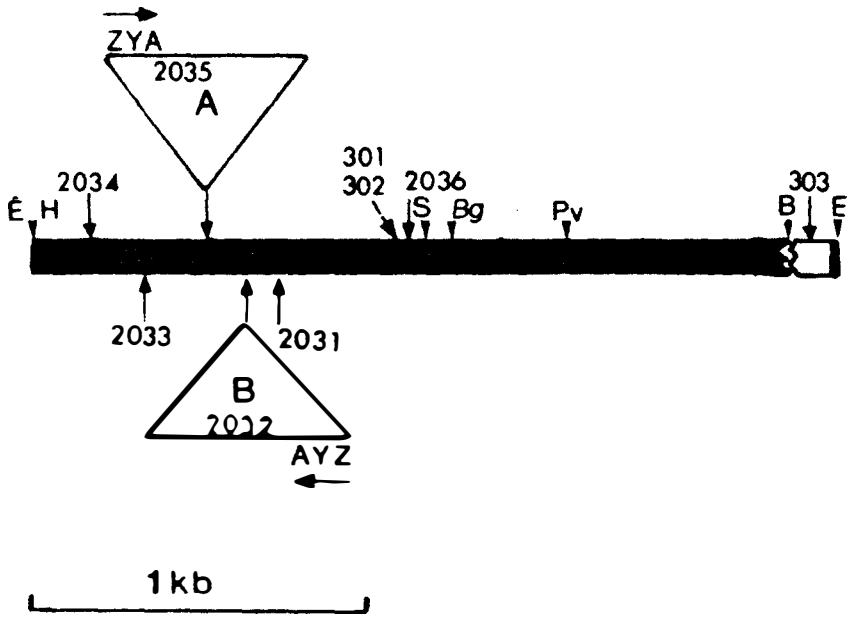
To more precisely identify the site of the recA-complementing activity, Tn5 insertion derivatives of pKML2003 were generated and physically mapped. Several independently isolated clones which had lost the MMS and NF resistance phenotype normally conferred by the plasmid were examined. All were found to contain an insertion in the same region of the plasmid and in the same orientation (Figure 5). Mapping indicated that the sites of Tn5 insertion were not distinguishable and have been assumed to represent insertions into the same site. One of these plasmids, pKML302, was utilized for further experimentation. The Tn5 insertion present in pKML302 caused the simultaneous loss of all activities associated with a $RecA^+$ phenotype in recA mutants of E. coli (see below).

Mini-Mu d(Kan^r, lac) fusions with the recA analogue.

To further localize the recA-complementing sequences and to determine the direction of transcription of the recA analogue, several independent Mini-Mu d(Kan^r,

lac) fusions in both orientations were isolated in pKML2003 (Figure 7). Two independently-generated clones representing Mini-Mu insertions in opposite orientations were introduced into E. coli JM103 to determine which construction was transcriptionally active, i.e., expressed β-galactosidase activity. It was determined that both constructions expressed β-galactosidase at very low levels. Since these Mini-Mu phages make translational fusions, it was possible that the fusion tested was not in the proper reading frame. In these constructions lacY is translated regardless of the reading frame generated by the fusion event since it has an independent translational start site. We therefore assayed the transcriptional activity of the fusion by testing for lacY activity. The product of the lacY gene is required by E. coli for growth at 42°C on medium with melibiose as the sole carbon source (129). Testing for growth on M9 melibiose agar revealed that inserts with orientation B expressed lacY at levels sufficient for the growth of JM103 under these culture conditions. This indicates that lacY is transcribed in fusion orientation B. Moreover, the lacY gene must be expressed at a minimum of 8% of the wild-type level to allow cell growth under these conditions (94).

Figure 7. Location of Mini Mu dII1681 insertions in pKML2003 P. aeruginosa chromosomal DNA which eliminate recA-complementing activity. Downward arrows denote insertions of orientation A with lac transcription from left to right on the map. Upward arrows denote isertions of orientation B with lac transcription proceeding from right to left. Insertions in orientation B were found to be transcriptionally active (see text). The positions of Tn5 insertions into the recA gene are also shown on this map (pKML301, 302 and 303). Plasmids pKML301 and pKML302 are Tn5 insertion derivatives of pKML2 and pKML2003 respectively which eliminate recA complementing activity. Plasmid pKML303 is a Tn5 insertion derivative of pKML2003 with the recA complementing activity intact and the ampicillin resistance activity abrogated.


ZYA
2035
A
2034
E H
301
302
2036
S
Bg
Pv
303
B
E
2033
2031
B
2022
AYZ
1kb

Determination of UV irradiation sensitivity.

The ability of the subclones to restore resistance to UV irradiation in E. coli recA mutants was determined. pKML2003 and pKML2004 were found to restore the UV resistance of strains containing recA mutations (Figure 8). Plasmid pKML302 was unable to confer resistance to UV irradiation to recA strains containing it.

Determination of conjugational proficiency.

To test recombinational proficiency of clones containing the P. aeruginosa recA analogue, matings between the HfrH strain JC158 and RecA$^-$ recipients were carried out. Plasmids pKML2003 and pKML2004 restored the homologous recombination proficiency following conjugation of various E. coli recA mutants to nearly wild-type levels (Table 9). The Tn5 insertion derivative of pKML2003, pKML302, was unable to support homologous recombination in these recA mutants.

Prophage induction.

In E. coli, the induction to lytic growth of resident lambda prophage subsequent to DNA damage requires the presence of a functional and specifically activated recA gene product (109,111). The ability of the P. aeruginosa recA analogue-containing clones to support spontaneous and MMC-stimulated induction of

Figure 8. Restoration of resistance by the P. aeruginosa recA analogue to killing by UV irradiation of recA mutants of E. coli. Cells were grown to a density of approximately 10^8/ml in Luria Broth, pelleted, suspended in saline, and exposed to various fluences of UV irradiation. After appropriate dilution in saline, the irradiated cells were plated in duplicate on L-agar and incubated at 37°C overnight in the dark. Mean and range of values are plotted. (●) E. coli HB101 (recA13); (○) E. coli RM2312 (recA13, pKML302); (△) E. coli JC2926 (recA13); (▲) E. coli RM2316 (recA13, pKML2003); (■) E. coli RM2317 recA13, pKML2004); (□) E. coli AB1157 (Rec^+). Plasmids pKML2003 and 2004 contain the P. aeruginosa recA complementing activity in opposite orientation relative to pBR322. Plasmid pKML302 is a Tn5 insertion derivative of pKML2003 with the recA activity abrogated.

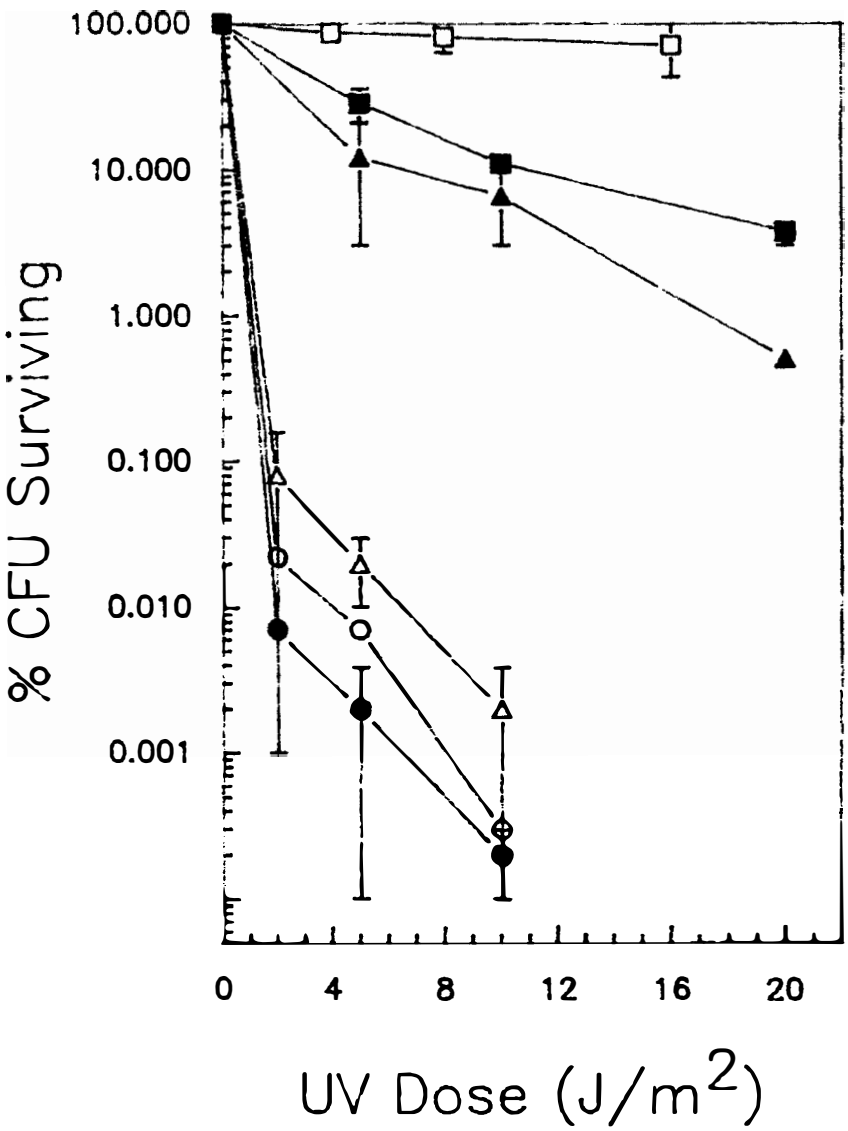

100.000
10.000
1.000
0.100
0.010
0.001
% CFU Surviving
0
4
8
12
16
20
UV Dose (J/m2)

Table 9. Recombinational Proficiency in Hfr Crosses[a].

Recipient	Relevant Characteristics[b]	Recombinational proficiency (*proA*$^+$ recombinants/100 donors)	
HB101	*recA13*	(1)[c]	3.5×10^{-5}
		(2)	$<4 \times 10^{-4}$
RM2310	*recA13*; pKML2003	(1)	0.75
		(2)	0.1
RM2312	*recA13*; pKML302	(1)	$<3 \times 10^{-6}$
		(2)	$<4 \times 10^{-4}$
AB1157	Rec$^+$	(1)	7.3
		(2)	4.3
JC2926	*recA13*	(1)	4.4×10^{-4}
		(2)	3×10^{-4}
RM2316	*recA13*; pKML2003	(1)	2.9
		(2)	3
RM2317	*recA13*; pKML2004	(1)	3.0

[a] *E. coli* JC158 was used as the donor strain. Cells were mixed in a donor-to-recipient ratio of 1:10. Matings were performed for 2 h at 37°C. Data is not corrected for possible F' formation.

[b] pKML2003 and pKML2004 contain the *P. aeruginosa recA* analogue. pKML302 was derived from pKML2003 and contains a Tn*5*-insertionally inactivated form of the *P. aeruginosa recA* analogue.

[c] Experiments were done twice except where indicated. All dilutions were plated in duplicate.

lambda prophage was investigated (Table 10). The presence of the P. aeruginosa recA analogue stimulates lambda prophage induction greatly. The ability of pKML2003 to support induction of a lambda cI ind prophage (λ207) was also tested. The P. aeruginosa recA analogue is unable to induce this lambda cI ind prophage to lytic growth following MMC treatment (Table 10).

Southern analysis of the recA analogue.

Nick translated pKML2 DNA was used to probe a blot of pJC859 DNA. This plasmid contains the E. coli recA gene on a 3.3 kb fragment of DNA cloned into the BamHI site of pBR322 (A. J. Clark, personal communication). Probing the blot under high stringency conditions demonstrated the existence of DNA base sequence homology between the E. coli recA gene and plasmid pKML2 (Figure 9). In order to be detected under these conditions, regions of plasmid pKML2 must be minimally 85% homologous to the E. coli recA gene DNA sequences.

Northern analysis of the recA analogue.

The expression of the recA analogue in P. aeruginosa was verified by Northern blotting experiments. The recA analogue is expressed in P. aeruginosa and probing the blot under conditions of high stringency with the 1.5 kb probe pKML2005, revealed the presence of two

Table 10. Lambda prophage induction.

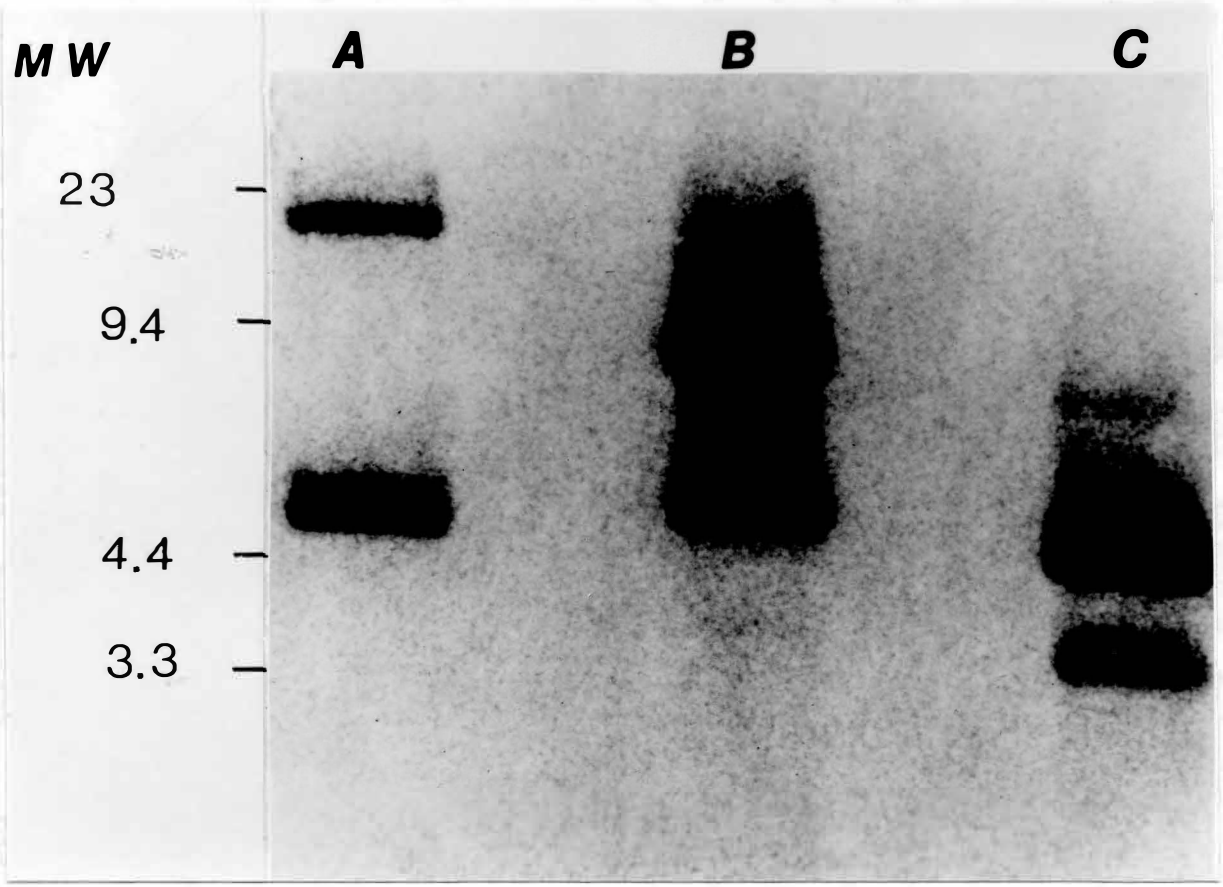
Strain	Relevant Characteristics		Viability[a] (CFU/ml	Phage produced (PFU/10^6 CFU)	
				Spontaneous	Induced[b]
λR Lysogens					
RM1184	Rec$^+$	(1)[c]	2 x 10^8	2,000	10,000,000
RM1186	recA56	(1)	6 x 10^7	1	1
		(2)	6 x 10^7	1	1.5
RM2318	recA56	(1)	5 x 10^7	800	2,000,000
	pKML2003[c]	(2)	5 x 10^7	1,200	1,000,000
λ 207 Lysogens					
RM1185	Rec$^+$	(1)	9 x 10^7	0.4	0.4
		(2)	9 x 10^7	0.1	0.1
RM2319	recA56	(1)	6 x 10^7	3	7.5
	pKML2003[c]	(2)	6 x 10^7	1.5	5

[a]Cells were incubated with mitomycin C at 5 ug/ml for 150 min.

[b]Experiments were done two times except where indicated.

[c]pKML2003 contains the *P. aeruginosa recA* analogue.

Figure 9. Southern analysis of the P. aeruginosa recA analogue-containing DNA. Plasmid pKML2 was nick translated and used to probe a Southern blot of plasmids containing the P. aeruginosa recA analogue or the E. coli recA gene. Plasmid DNAs were digested with BamHI before electrophoresis. Hybridization was carried out under conditions of high stringency (see text). (A) pKML1; (B) pKML2; (C) pJC859. Plasmids pKML1 and pKML2 contain the P. aeruginosa recA complementing activity on approximately 25 and 9.2 kb fragments of P. aeruginosa DNA respectively. Plasmid pJC859 contains the authentic E. coli recA gene on an approximately 3.3 kb fragment of DNA.


MW
A
B
C
23
9.4
4.4
3.3

distinct mRNA bands (Figure 10) with sizes of 2.8 and 1.6 kb.

Identification of the protein product of the P. aeruginosa recA analogue.

Minicells were used to estimate the size of the P. aeruginosa recA analogue protein product (Figure 11). Comparison to size markers run in parallel revealed that the protein has a molecular weight of approximately 47,000. A phenotypically Rec^- deletion derivative of pKML2003 with the sequences between the BamHI and BglII sites removed (pKLM2006) did not produce this protein in minicells (Figure 11). A Tn5 insertion derivative of pKML2, pKML301, also does not produce this protein in minicells (data not shown). Plasmid pKML2004, which contains the BamHI-XhoI fragment of pKML2, was found to produce a protein of the same molecular weight as pKML2003.

Induction of dinB1::Mud(Amp^r, lac) expression.

Transcription of the dinB gene has been shown to be inducible by UV irradiation (79). This induction of expression depends upon the recA gene product to promote cleavage of the lexA-encoded repressor. Induction of this gene can be monitored by assaying β-galactosidase activity in a dinB1::Mud(Amp^r, lac) fusion mutant. The P. aeruginosa recA analogue was capable of allowing the UV

Figure 10. Northern analysis of *P. aeruginosa recA* analogue. Plasmid pKML2003 was nick translated and used to probe a Northern blot of RNA extracted from *P. aeruginosa* PAO1 as described in the text. Sizes are in kilobase pairs. Plasmid pKML2003 contains the *P. aeruginosa recA* gene on a 2.3 kb DNA fragment.

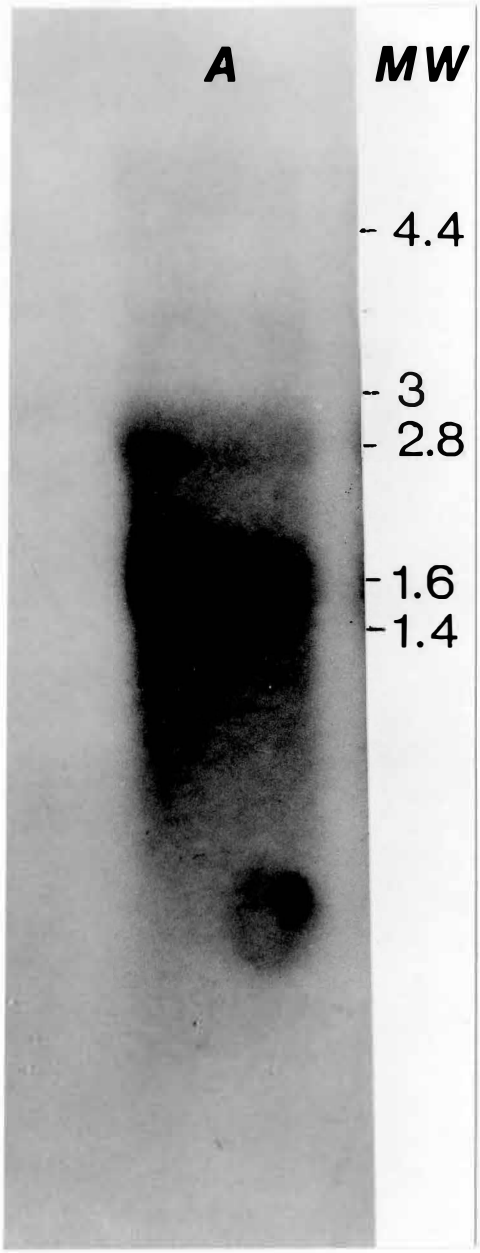
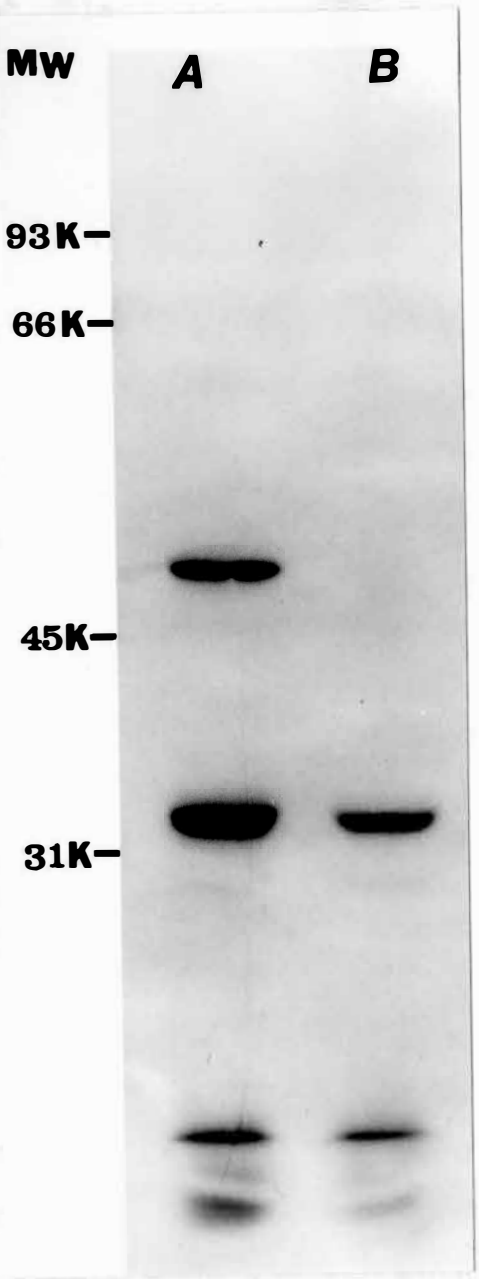

A
MW
4.4
3
2.8
1.6
1.4

Figure 11. Identification of the *P. aeruginosa* *recA* analogue gene product. [^{35}S]-labeling of plasmid encoded proteins was carried out as described in the text. (A) *E. coli* RM2325 which contains pKML2003. Plasmid pKML2003 contains the *P. aeruginosa* *recA* gene on a 2.3 kb *BamHI-HindIII* fragment of DNA. (B) *E. coli* RM2326 which contains pKML2006, a Rec$^-$ deletion derivative of pKML2003 with the *BamHI-BglII* fragment removed. Migration of strandard molecular weight markers is indicated to the left.


MW
A
B
93K
66K
45K
31K

induction of β-galactosidase expression in a recA56 dinB1::Mud(Amp^r, lac) mutant (Table 11). This induction of expression was specifically dependent upon UV irradiation to the cell, no induction of expression above the nominal baseline level was observed in the absence of exposure to UV irradiation.

UV sensitivity of RecA$^+$ E. coli containing Tn 5-inactivated or lacZ-fused recA analogue plasmids.

The presence of a plasmid containing a Tn5-inactivated recA analogue (pKML301, Figure 7) did not sensitize E. coli AB1157 to UV irradiation (Figure 12). However, the presence of plasmid pKML2031 (Figure 7) containing a fusion of lacZ to the recA analogue did result in the sensitization of E. coli JM103 to UV irradiation (Figure 13).

Discussion

A clone of P. aeruginosa PAO chromosomal DNA capable of complementing, in trans, E. coli recA mutant strains to UV resistance and recombinational proficiency has been isolated. The location of the recA complementing sequences was determined by subcloning analysis. A 2.3 kb DNA segment present in pKML2003

Table 11. Induction of *dinB1*::Mud(Amp^r,*lac*) expression in GW1031 with and without pKML303[a].

UV[b]	pKML303[c]	β-Galactosidase Activity (Units/A_{600} Unit)[d]		
		0 min	60 min	120 min
No	Absent	43	42	22
	Present	20±10	40±2	50±10
Yes	Absent	43	22	33
	Present	20±10	50±6	114±15

[a]Cells were grown in supplemented M9-glucose medium at 30°C.

[b]Cultures were divided into two aliquots and one was exposed to UV irradiation: 2 J/m^2 for GW1031(*recA56*) and 10 J/m^2 for RM1139 (GW1031 containing pKML303) at time 0.

[c]pKML303 contains the *P. aeruginosa recA* analogue.

[d]Samples were removed at the indicated times and β-galactosidase activity determined by the method of Miller (95). Cell density was determined by measuring the A_{600}. Values are averages of two determinations. Activity in RM1139 was measured in two experiments. Averages and ranges of values are reported.

Figure 12. UV irradiation resistance of *E. coli* containing pKML301. Cells were treated as detailed for Figure 8. Mean and range of values of two repetitions are plotted. (□) *E. coli* AB1157 (Rec^+); (●) *E. coli* RM5000 (Rec^+, pKML301); (△) *E. coli* JC2926 (*recA13*). Plasmid pKML301 is a Tn*5* insertion derivative of pKML2 with the *recA* complementing activity destroyed.

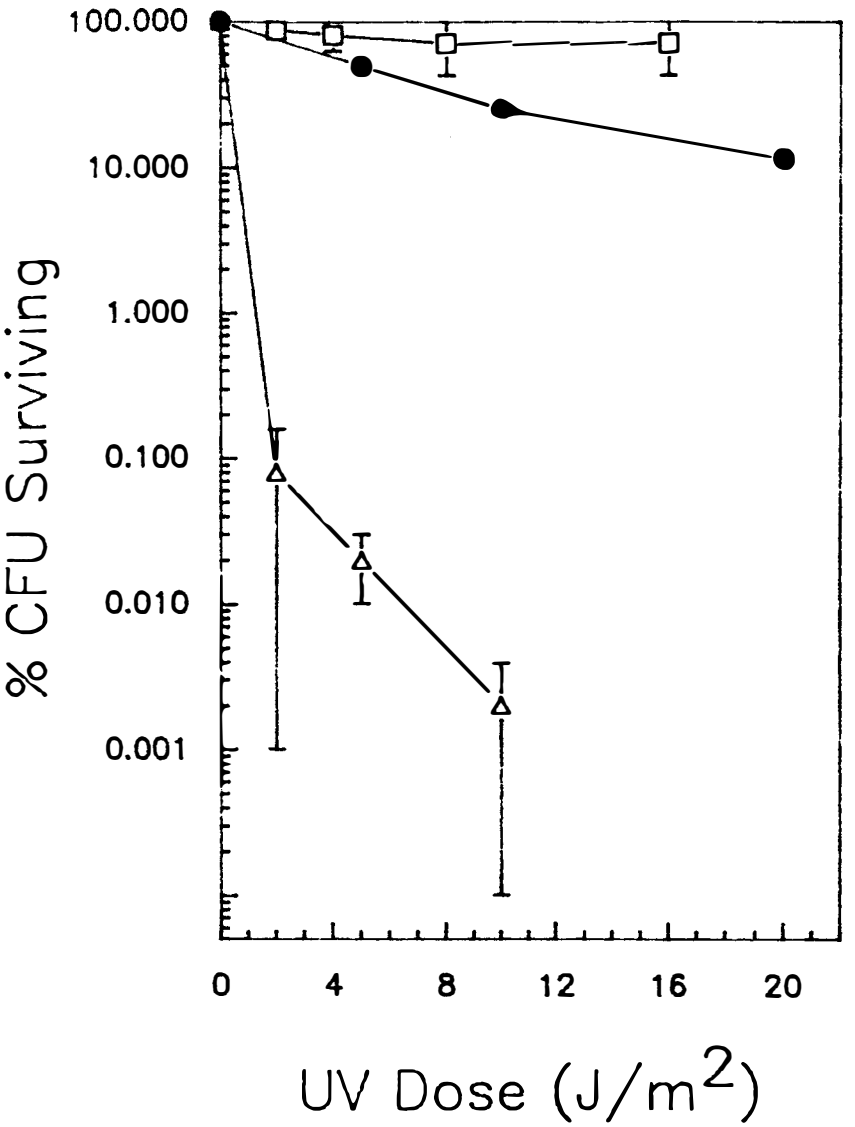
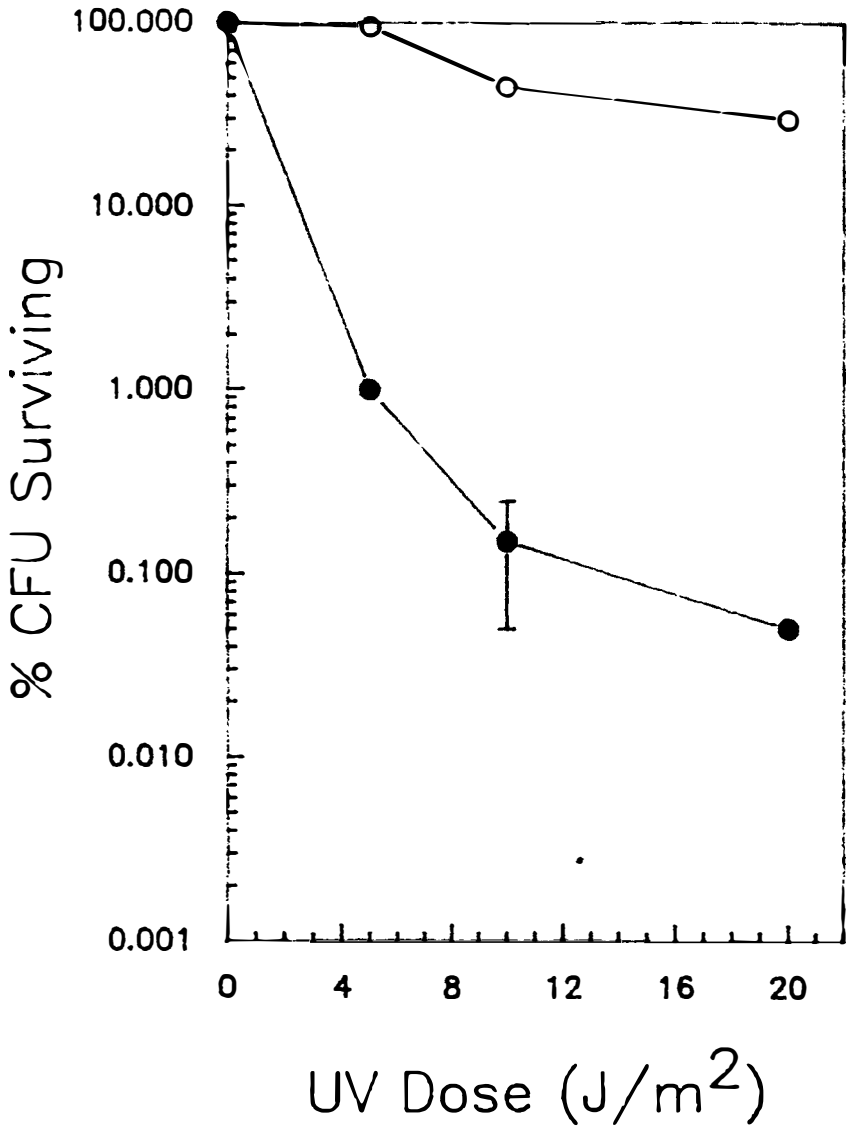

100.000
10.000
1.000
0.100
0.010
0.001
% CFU Surviving
0
4
8
12
16
20
UV Dose (J/m2)

Figure 13. UV irradiation resistance of *E. coli* containing pKML2031 (pKML2003::Mini-Mu d(Kan^r, *lac*). Cells were treated as detailed for Figure 8. Mean and range of values for two repetitions are plotted. (o) *E. coli* JM103 (Rec$^+$); (●) *E. coli* RM5001 (Rec$^+$, pKML2031).


100.000
10.000
1.000
0.100
0.010
0.001
% CFU Surviving
0
4
8
12
16
20
UV Dose (J/m2)

contains the entire recA analogue. That this segment contains the endogenous promoter of this gene is suggested by the observation that this gene is expressed when inserted in either orientation into pBR322.

Translational fusions of the recA analogue with lacZYA were constructed using Mini-Mu phage. The pattern of expression of such gene fusions suggests that the P. aeruginosa recA analogue is transcribed in the direction from BamHI toward HindIII on the physical map (Figure 5). In addition, the nucleotide base sequence of the P. aeruginosa recA analogue has been determined and is consistent with the direction of transcription in the same orientation as indicated by the gene fusions (M. Kageyama, personal communication). However, it was possible to examine only two fusion constructions so this determination of gene orientation must be regarded as tentative.

The E. coli recA gene product has been identified as a polypeptide of approximately 42,000 daltons by SDS-polyacrylamide gel electrophoresis (119). Examination of the DNA sequence of the recA gene has predicted a smaller molecular weight for the protein of 37,842 (120). Minicell analysis of the P. aeruginosa recA analogue indicates that its protein product is larger. A polypeptide of 47,000 daltons was produced in minicells

containing clones of the recA analogue inserted into the vector plasmid in either orientation indicating that the gene product is under control of its endogenous promoter in these constructions. Tn5-insertional inactivation of the P. aeruginosa recA analogue as well as the deletion of the BamHI-BglI fragment of pKML2003 specifically abolish the presence of this protein in minicell extracts, confirming that it is the product of the P. aeruginosa recA gene.

While no explanation for the difference in size between the predicted and observed size of the E. coli recA protein has been proposed, these findings suggest that the size of the P. aeruginosa recA analogue gene product may be an overestimate. SDS- polyacrylamide gel electrophoresis provides an accurate method for determination of the subunit molecular weight of proteins if certain precautions are observed (94). The proteins must be adequately denatured and for this excess SDS must be present in the sample buffer to prevent artifacts in size estimation. To perform the most accurate size estimation it must be ascertained that the proteins are migrating in a manner consistent with their size for several concentrations of acrylamide to detect artifacts in the migration of the protein. This was apparently not

done for the E. coli protein and was not done for the estimate of the P. aeruginosa recA analogue protein, so the size determined must be regarded as tenative.

Northern analysis of mRNA isolated from P. aeruginosa strain PAO1 has indicated that the chromosomal sequences contained in pKML2003 are expressed in P. aeruginosa PAO1. It is interesting that the Northern blot has detected two mRNA bands with homology to the probe. Probing with pKMKL2005 has likewise revealed the presence of two homologous mRNA species (data not shown). Either of these messengers would have sufficient size to encode the recA analogue protein product. The presence of two mRNA species could indicate the presence of multiple transcriptional start sites for the recA gene. Analysis of RNA transcripts of other P. aeruginosa genes has revealed that multiple transcripts are detected (M. Vasil and A. Chakrabarty, personal communications).

Southern analysis of pKML2 demonstrated DNA base sequence homology to the E. coli recA gene. Western analysis of the protein product of this gene expressed in E. coli JC13551, a recA deletion mutant, using anti-E. coli RecA antibody has revealed that the protein shares antigenic cross-reactivity with the E. coli RecA protein (S. Kowalczykowski, personal communication). The data reported here suggest that the recA gene, whatever its

origin, has been well conserved, both structurally and functionally.

Sedgwick and Yarronton (126) demonstrated that introduction of a plasmid containing a truncated *recA* gene into a RecA$^+$ strain of *E. coli* often increases the cell's sensitivity to UV irradiation. Their analysis demonstrated that this sensitization of RecA$^+$ *E. coli* strains to UV irradiation was dependent upon a minimum size for the truncated gene product. The introduction of pKML302 or pKML2006 into *E. coli* AB1157 does not result in the strain exhibiting increased sensitivity to UV irradiation. This suggests that the site of Tn*5* insertion in pKML302 and the *BamHI-BglII* restriction fragment deleted in pKML2006 may be close to the 5' region of the gene and thus generate truncated polypeptides incapable of causing radiosensitization of *E. coli* AB1157. This interpretation makes the assumption that truncated fragments of the *P. aeruginosa recA* protein behave in the same fashion as the *E. coli* polypeptides.

The presence of plasmid pKML2031 containing a fusion of *lacZ* and the *recA* analogue in *E. coli* JM103 causes the strain to become sensitive to UV irradiation (Figure 13). Translational fusions of the *E. coli recA*

gene with *lacZ* when contained in multicopy plasmids have been found to cause radiosensitization of RecA$^+$ *E. coli* strains (148). This has been interpreted to be an effect of the induction of high-level expression of the fusion protein resulting from the exposure of the cell to UV irradiation with the concomitant elimination of LexA repression of the fusion construction. The fusion protein is proposed to be lethal when expressed at high levels. The *P. aeruginosa* fusion construction must normally be expressed at a level allowing cell survival. Upon exposure to UV irradiation, the expression of the fusion protein is induced resulting in cell death. This observation suggests that the expression of the *P. aeruginosa recA* analogue may be controlled negatively in *E. coli*, presumably by the host *lexA* gene product. However, it is possible that the sensitization effect produced by the fusion plasmid is actually analogous to the effects produced by truncated RecA polypeptides discussed above. The sensitization produced may be due not to induction of a lethal fusion protein, but rather mixed multimer production which interferes with wild-type functions. On the basis of the experiments described above, it is impossible to conclude that the expression of the *P. aeruginosa recA* analogue is inducible in the *E. coli* genetic background. However, the DNA nucleotide

base sequence of the P. aeruginosa recA analogue has revealed a potential SOS box (LexA binding region) at the promoter (M. Kageyama, personal communication).

The cloned P. aeruginosa recA analogue is capable of induction of resident lambda prophage to lytic growth in response to DNA damaging agents. It also allows the expression of β-galactosidase from the dinB1::Mud(Amp^r, lac) fusion. Hence, the recA-complementing activity is capable of inducing the expression of genes under control of either the cI- or lexA-encoded repressors. A number of lambda phage mutants have been isolated that are not inducible by agents such as UV irradiation or MMC (47). One such uninducible mutant has been shown to encode a cI repressor resistant to recA-mediated cleavage (111,112). We have demonstrated that the P. aeruginosa recA analogue is incapable of inducing the lytic growth of a resident uninducible lambda prophage. This suggests that the mechanism of elimination of cI-mediated repression of lytic functions of prophage lambda is likely to be very similar or identical for both the E. coli and P. aeruginosa recA gene products.

The P. aeruginosa recA analogue is clearly capable of the induction of expression of a din gene in the E. coli genetic background. It may then be inferred that it

is likely to allow the induction of expression of all din genes. It is unknown whether a lexA analogue exists in P. aeruginosa. If a homologous analogue of the lexA gene is expressed in P. aeruginosa, the recA analogue would be capable of inactivating any repression of gene expression mediated by the P. aeruginosa LexA protein.

It is clear that not all species of gram-negative bacteria possess an inducible system of error-prone DNA repair (10,125). Error-prone DNA damage repair mechanisms are not induced by nalidixic acid (10) in P. aeruginosa. Other studies have demonstrated that error-prone repair is not induced upon exposure of P. aeruginosa PAO1 to UV irradiation as well (R. V. Miller, personal communication). It is unknown if an error-free system of DNA repair is induced by exposure of the cell to UV or nalidixic acid. These data indicate that while the recA gene has been well conserved, some species, including P. aeruginosa, may possess systems with different potentials for DNA repair and mutagenesis.

CHAPTER IV

CHARACTERIZATION OF *P. AERUGINOSA RECA* MUTANTS

P. aeruginosa PAO1 chromosomal DNA sequences capable of complementing the pleiotropic effects of various *E. coli recA* mutations have been identified from a random library of *P. aeruginosa* PAO1 chromosomal DNA described in Chapters II and III. The ability of the cloned *P. aeruginosa recA* analogue to be expressed and function in *E. coli* implies that functional aspects of the *recA* gene have been conserved among the Gram negative bacteria. It is therefore possible that the *recA* analogue executes the same functions in *P. aeruginosa* as the *recA* gene product does in *E. coli*. If so, a *recA* mutant of *P. aeruginosa* should exhibit a greater sensitivity to ultraviolet (UV) irradiation and agents that damage DNA such as Mitomycin C (MMC) and methyl methanesulfonate (MMS). *P. aeruginosa recA* mutants would be expected to show a greatly reduced ability to support

homologous recombination. In addition, it seems likely that lysogens of temperate phages of P. aeruginosa capable of UV induction to lytic growth from the prophage state, such as phages D3 and F116L (62), would require the presence of a functional recA analogue.

Several Rec$^-$ mutants of P. aeruginosa PAO have been described which exhibit a number of these characteristics (23,43,60,64,96). Früh, et al., reported in 1983 the isolation and characterization of several Rec$^-$ strains of Pseudomonas aeruginosa PAO (43). These strains were constructed by conjugation with Rec$^-$ strains of nitrosoguanidine-mutagenized P. aeruginosa PAT. These strains were found to be markedly deficient in the ability to recombine DNA received by either transduction or R68.45-mediated conjugation, more sensitive to UV irradiation than the parental strain, and sensitive to the presence of MMC in the growth medium. The allele contained in these strains, rec-102, was mapped to approximately 42 minutes on the P. aeruginosa PAO chromosomal map. Miller and Ku reported the isolation of a number of mutant strains of P. aeruginosa PAO deficient in the establishment of lysogeny (96). One of the mutations isolated, lesB908, has several properties suggesting that it may be analogous to recA mutations of E. coli. Strains containing lesB908 are sensitive to UV

irradiation and are unable to undergo homologous recombination. This mutation was mapped to the 40-45 minute region of the PAO chromosomal map. In addition, Ohman, et al., constructed a recA mutant of the clinical isolate P. aeruginosa FRD. This strain is unable to support homologous recombination and is much more sensitive to UV irradiation than the parental strain (101). The chromosomal locus of this mutation has not been determined.

The 2.3 kb fragment of the P. aeruginosa PAO chromosome which complements recA mutations of E. coli in trans described in the previous chapters was subcloned into the broad-host-range plasmid pCP13 (31) and mobilized into P. aeruginosa rec-102-containing strains. The effect of this plasmid upon the pleiotropic phenotype conferred by the rec-102 mutation are described in this Chapter.

Materials and Methods

Bacterial and bacteriophage strains.

The bacterial strains used are listed in Table 12. Phage D3 is a specialized transducing temperate phage of P. aeruginosa (21). D3c is a spontaneous clear-plaque

Table 12. Bacterial strains.

Strain	Plasmid	Relevant genotype[a]												Other markers	Prophage[a]	Source or reference
		rec	arg	his	hsd	ilv	leu	lys	pro	pur	sup	trp	thi			
E. coli																
HB101		A13	+	+	S20	+	-	+	A2	+	E44	+	-1			(13)
RM2320	pRML3001	A13	+	+	S20	+	-	+	A2	+	E44	+	-1			HB101
RM5002	pCP13	A13	+	+	S20	+	-	+	A2	+	E44	+	-1			HB101
P. aeruginosa																
PAO1		+	+	+	+	+	+	+	+	+	+	+	+			(96)
PAO25		+	F10	+	+	+	-10	+	+	+	+	+	+			(43)
PAO303		+	B21	+	+	+	+	+	+	+	+	+	+			(96)
PAO832	FP5	+	+	-151	+	-261	+	+	+	-66	+	+	+	*pyr-21*		(96)
PAO4141		+	+	+	+	+	+	+	-9024	+	+	+	+	*met-9020*	*aph-9001*	H. Matsumoto
PTO66		-102	+	-4	+	-1118	+	-12	-82	+	+	-6	+	*fon*		(43)
PTO6003		-102	+	+	+	+	+	+	+	-67	+	+	+			(43)

(Table 12: Continued)

Strain	Plasmid	Relevant genotype[a]												Other markers	Prophage[a]	Source or reference
		rec	arg	his	hsd	ilv	leu	met	pro	pur	sup	thr	thi			
RM187	FP5	+	B21	+	+	+	+	+	+	+	+	+	+	nalA901		PAO25
RM247		+	F10	+	+	+	-10	+	+	+	+	+	+		(D3)	PAO25
RM265		-102	+	+	+	+	-10	+	+	+	+	+	+			PAO25
RM276		-102	+	+	+	+	-10	+	+	+	+	+	+		(D3)	RM265
RM2321	pRML3001	-102	+	-4	+	-1118	+	-12	-82	+	+	-6	+			PTO66
RM2322	pRML3001	-102	+	+	+	+	+	+	+	-67	+	+	+			PTO6003
RM2323	pRML3001	-102	+	+	+	+	-10	+	+	+	+	+	+			RM265
RM2324	pRML3001	-102	+	+	+	+	-10	+	+	+	+	+	+		(D3)	RM265
RM9999		-5::Tn5	+	+	+	+	+	+	-9024	+	+	+	+	met-9020 aph-9001		PAO4141

[a]Genotype symbols are as described in Demerec et al. (38). Abbreviations are as specified by Bachmann (3) except fon which indicates resistance to phage F116L. Prophage are symbolized by including the name of the phage in parenthesis when present.

mutant of D3. F116L is a temperate generalized transducing phage of P. aeruginosa (61).

Plasmids.

Plasmid pCP13 is a derivative of the broad-host-range cosmid pLAFR1 (31,42). Plasmid pKML3001 contains the P. aeruginosa recA analogue on an approximately 2.3 kb BamHI-HindIII DNA fragment, replacing the BamHI-HindIII fragment of pCP13.

Media and chemicals.

Cells were grown in LB at 37°C. Antibiotics were used at the following concentrations: ampicillin, 50 ug/ml; kanamycin, 50 ug/ml; tetracycline, 12.5 ug/ml for E. coli. For P. aeruginosa tetracycline was used at 250 ug/ml. PMM (Chapter II) containing 0.4 % (w/v) glucose and supplemented with appropriate amino acids (25 ug/ml) was used for conjugation experiments. Lysates of bacteriophages were prepared on L-agar and phage were plated in lambda top agar as described in Chapter II. Pseudomonas Isolation Agar was purchased from Difco.

Restriction endonucleases were used as described in Chapter II.

Bacterial conjugations.

The procedure used was a modification of the method of Okii, et al. (102). Cells were grown to mid-log phase in LB at 37°C with agitation. The cells were harvested

by centrifugation and suspended in an equal volume of LB. The cells were mixed in the ratio of 1 ml of donor culture to 1.2 ml of recipient culture and incubated without shaking for 2 h at 37°C. The conjugation mix was plated on PMM selective agar. Selection of recombinants was by aquisition of amino acid prototrophy and contraselection of donor cells was by amino acid auxotrophy. For the determination of the acquisition of plasmid FP5, the mating procedure was identical to that described above, however, transconjugants were selected for resistance to $HgCl_2$ (2.5 ug/ml).

Mobilization of pKML3001 from E. coli to P. aeruginosa.

Triparental matings were performed using a modification of the method of Ruvken and Ausubel (118). Cultures of E. coli HB101 containing the mobilizing plasmid pRK2013 (42) or pKML3001 were grown to saturation in LB at 37°C. P. aeruginosa strains to be mated were grown 16 h at 43°C and concentrated five-fold before conjugation. These conditions allowed a more efficient introduction of plasmid DNA into P. aeruginosa as the restriction system of P. aeruginosa PAO is phenotypically disabled (115). Cells were conjugated by mixing 0.1 ml amounts of each of the three strains and spreading the

mixture over an area of 4 cm^2 on L-agar plates. Mating was allowed to proceed for 8 h at 37°C. The cells were harvested by washing the plate with LB and the washings plated on Pseudomonas Isolation Agar supplemented with 250 ug of tetracycline/ml of medium. The plates were incubated for 1-2 days at 37°C.

Southern analysis of chromosomal DNA.

Chromosomal DNA (5 ug) isolated as detailed in Chapter II was digested with BamHI or HindIII and PvuII and electrophoresed on a 0.7% agarose gel (33). This DNA was blotted onto nitrocellulose filters (BA85, 0.45 um pore size; Schleicher and Scheull, Keene, NH) using capillary transfer (86). Plasmid pKML2003 was labeled with [α-^{32}P]-dCTP (800 Ci/mM; New England Nuclear, Boston, MA) by nick translation using a kit obtained from Amersham Corp. (Arlington Heights, IL). Separation of labeled probe from unincorporated nucleotides was by the spun column procedure of Maniatis, et al. (86). The labeled plasmid DNA was used to probe the blotted chromosomal DNA. Conditions for hybridization and washing were as described by Wahl, et al. (140). The blot was prehybridized in a solution of 50% (v/v) formamide, 5 X SSC, 5 X Denhardt's solution and 250 ug denatured calf thymus DNA/ml for 60 min at 42°C. The hybridization solution was four parts prehybridization

solution and one part 50% (w/v) Dextran sulfate. Addition of labeled probe was handled as in Chapter III. Hybridization was allowed to proceed 16 at 42°C. At the conclusion of the hybridization period, the blot was washed three times in 250 ml of 2X SSC containing 0.1% (w/v) SDS at room temperature for 5 min. The blot was then washed twice in 250 ml 0.1X SSC containing 0.1% (w/v) SDS at 45°C for a total time of 30 min. After this treatment, the blot was air dried and autoradiographed.

Phage induction.

Cells to be used for induction studies were grown to approximately 2 x 10^8 CFU/ml in LB. The cells were harvested by centrifugation at 5,000 x g for 5 min. The cells were suspended in an equal volume of saline and exposed to UV irradiation at a fluence of 10 J/m^2. The irradiated cells were incubated in the dark for 2 h and lysed by addition of a 1/10 volume of chloroform. The cells were centrifuged as above to remove debris and the lysates titered for phage. P. aeruginosa PAO1 was used as the indicator strain with titration done using a soft agar overlay technique (2).

Construction of recA::Tn5 P. aeruginosa strains.

Plasmid pKML302 was mobilized into P. aeruginosa PAO4141 using the triparental mating technique described

above. Selection was made for transconjugants using pseudomonas isolation agar containing kanamycin at 10 ug/ml. Strain PAO4141 is kanamycin supersensitive (H. Matsumoto, personal communication). Kanamycin resistant transconjugants were examined for the acquisition of UV irradiation sensitivity and recombination deficiency.

F116L transductions.

Strains to be transduced were grown to a density of 10^8 cfu/ml in LB. Cells were harvested by centrifugation and suspended in an equal volume of 10 mM Tris (pH 8), 10 mM $MgCl_2$ and 10 mM NaCl. Cells were infected at several different moi's, incubated at 37°C for 30 min and plated on PSM medium. Selection was made for methionine prototrophy.

Other methods.

Isolation of chromosomal DNA and determination of UV sensitivity were done as detailed in Chapter II.

Results

Southern analysis of a rec-102 containing strain.

Chromosomal DNA from P. aeruginosa was digested with BamHI and HindIII or XhoI, electrophoresed in a 0.7% agarose gel and blotted to nitrocellulose. The blot was probed with nick-translated pKML2003 DNA. Several bands

are homologous to the probe DNA (Figures 14 and 15). The smallest band observed is of the size predicted for a BamHI-HindIII fragment representing the recA coding region of P. aeruginosa. The presence of multiple hybridizing bands were probably produced because the restriction digestion was not complete but, a partial digest. Utilizing recA-containing R' plasmids this region of the chromosome has been restriction endonuclease mapped (M. Kageyama personal communication). On the basis of this mapping data, the extra hybridizing bands present in the Southern blot of PAO25 DNA are predictable as partial digestion products.

The original isolation of the rec-102 mutation was done using nitrosoguanidine mutagenesis (43). It therefore seemed possible that a number of encoding nucleotides had been altered which might lead to changes in the restriction map of the chromosomal region encoding the altered gene. Chromosomal DNA from PAO25 (Rec^+) and a rec-102-containing derivative of PAO25, RM265, was isolated, digested with various restriction endonucleases, and Southern blotted onto nitrocellulose. The blots were probed with nick-translated pKML2003 DNA which contains the P. aeruginosa PAO recA analogue (Chapters II and III). The Southern analysis revealed a potential restriction endonuclease polymorphism in the

Figure 14. Ethidium bromide staining of restriction digests of P. aeruginosa chromosomal DNA. Chromosomal DNA was digested with restriction endonucleases and electrophoresed on 0.7% agarose gels. A: BamHI-HindIII digest. B:XhoI digest. MW: Molecular weight markers.

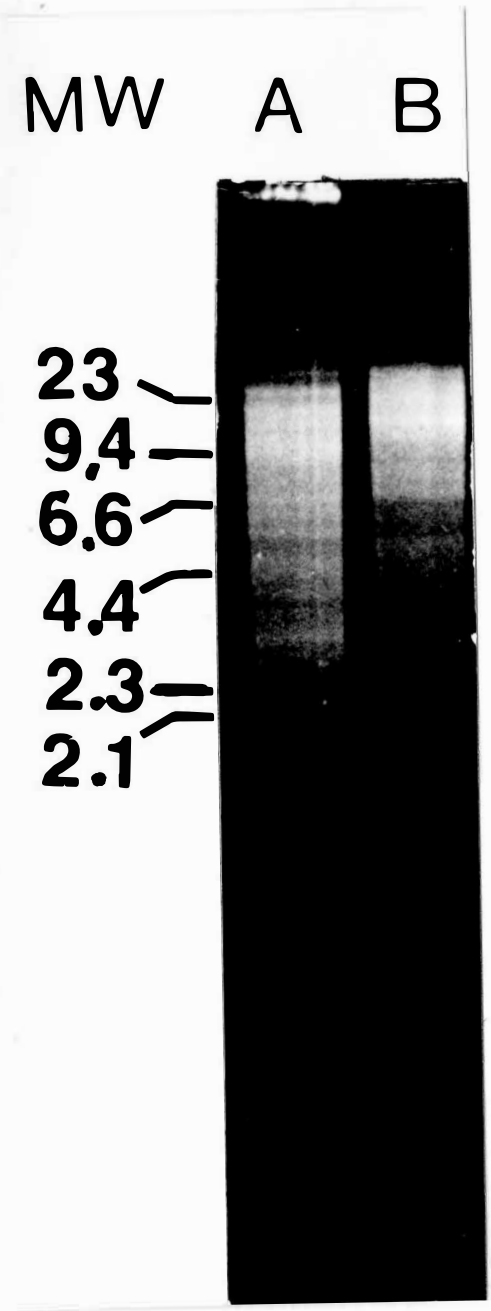
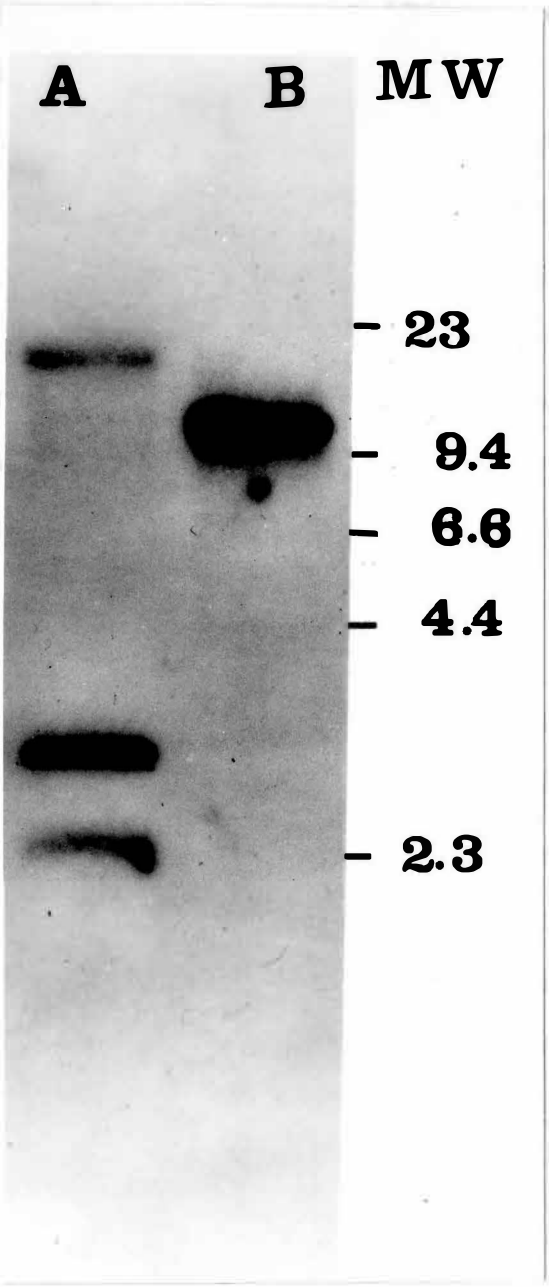

MW
A
B
23
9.4
6.6
4.4
2.3
2.1

Figure 15. Southern analysis of P. aeruginosa chromosomal DNA probed with pKML2003. Gel shown in Figure 14 was Southern blotted to nitrocellulose filters as described in text. A: BamHI-HindIII digest. B: XhoI digest. MW: Molecular weight markers.


A
B
MW
23
9.4
6.6
4.4
2.3

HindIII-PvuII restriction pattern between the wildtype and rec-102-containing clones (Figure 16). HindIII and PvuII sites either closely bracket the P. aeruginosa recA analogue or occur within the gene (Chapter III). It therefore seemed possible that rec-102 was a mutant allele of the P. aeruginosa recA analogue contained in pKML2003. It is also possible that the polymorphism detected represented a difference between P. aeruginosa PAO and PAT DNA. To determine if rec-102 is in fact a mutant allele of the P. aeruginosa recA gene a broad-host-range plasmid which contains the P. aeruginosa PAO recA analogue was constructed and its ability to complement the rec-102 phenotype tested.

Construction of pKML3001.

The 2.3 kb HindIII-BamHI fragment of the PAO1 chromosome from pKML2003 was cloned into the broad-host-range vector pCP13 (Figure 17). This construction was transformed into E. coli strain HB101 and clones were screened for resistance to NF. Positive isolates were shown to contain plasmid DNA of the appropriate restriction pattern (data not shown). The ability of the selected clones to complement the defect in UV repair associated with the recA mutation of HB101 was confirmed (Figure 18). The plasmid from one such clone, pKLM3001, was mobilized into several rec-102 containing P.

Figure 16. Southern analysis of Rec^+ and Rec^- isogenic strains of P. aeruginosa. Chromosomal DNA was digested with BamHI alone or both HindIII and PvuII and blotted onto nitrocellulose. This blot was probed with nick translated pKML2003 DNA. Hybridization was carried out under conditions of high stringency. (A) PAO25 DNA, BamHI digest; (B) RM265 DNA, BamHI digest; (C) PAO25 DNA, HindIII-PvuII digest; (D) RM265 DNA, HindIII-PvuII digest.

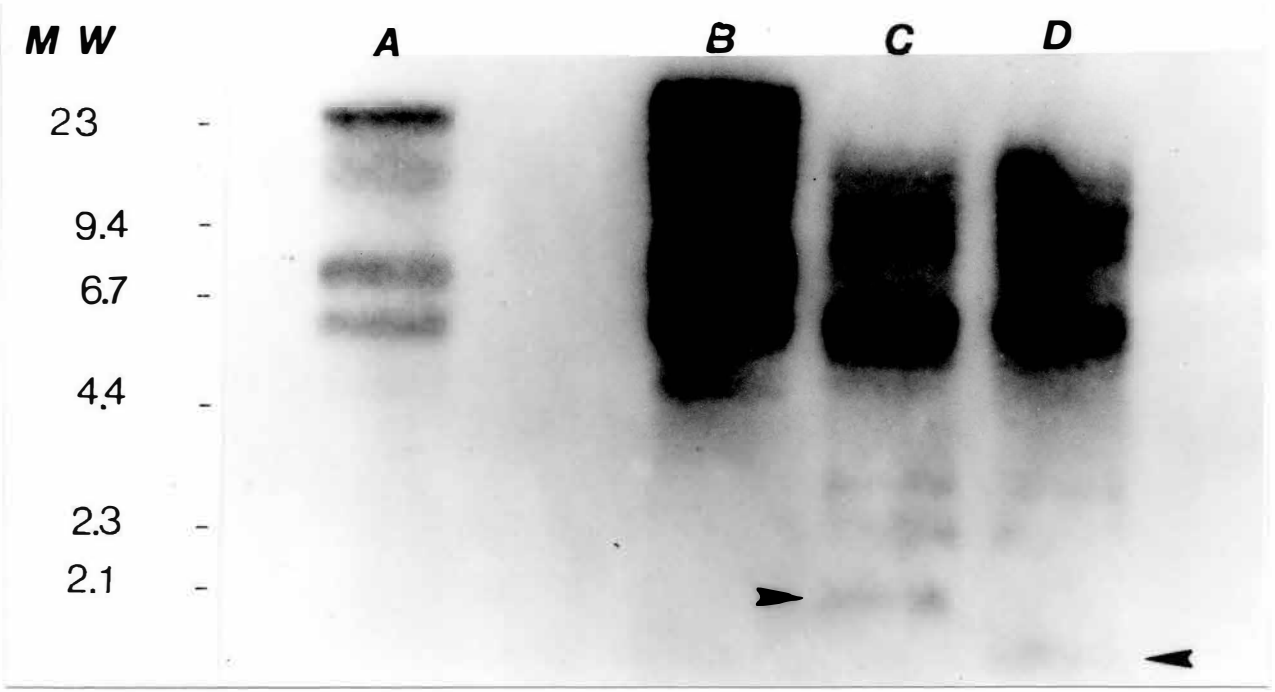

M W
A
B
C
D
23
9.4
6.7
4.4
2.3
2.1

Figure 17. Restriction endonuclease map of pKML3001. The thick insert line represents P. aeruginosa chromosomal DNA. The thin line represents pCP13 DNA. The cos packaging site is represented by the solid box. Symbols: B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; S, SalI; Tc^r, tetracycline resistance gene; cos, cos packaging site.

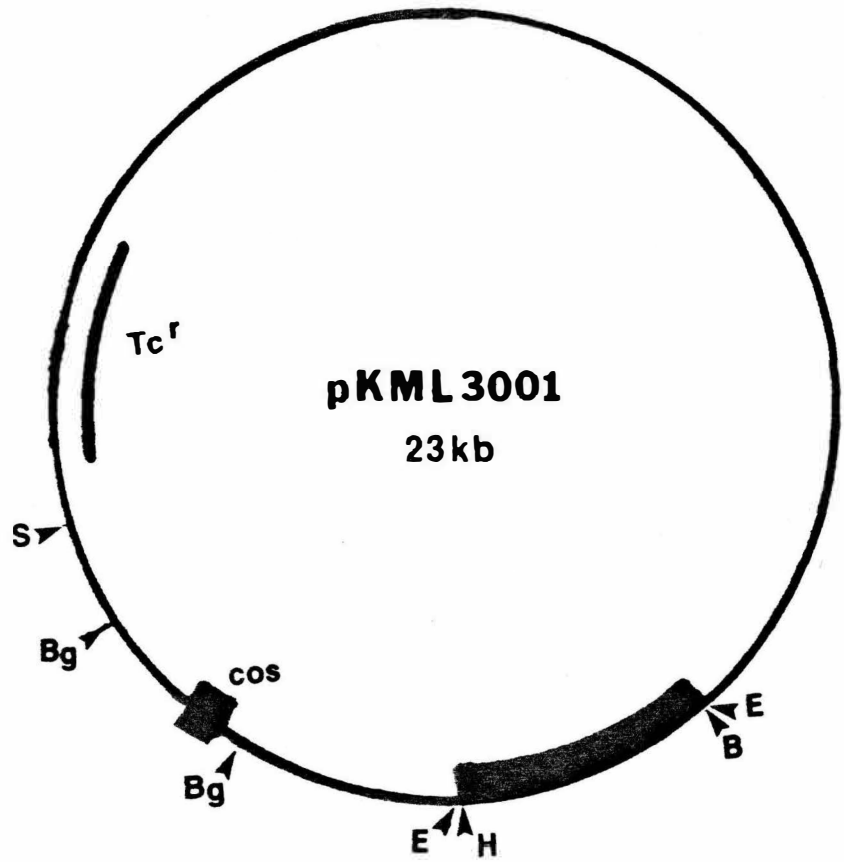
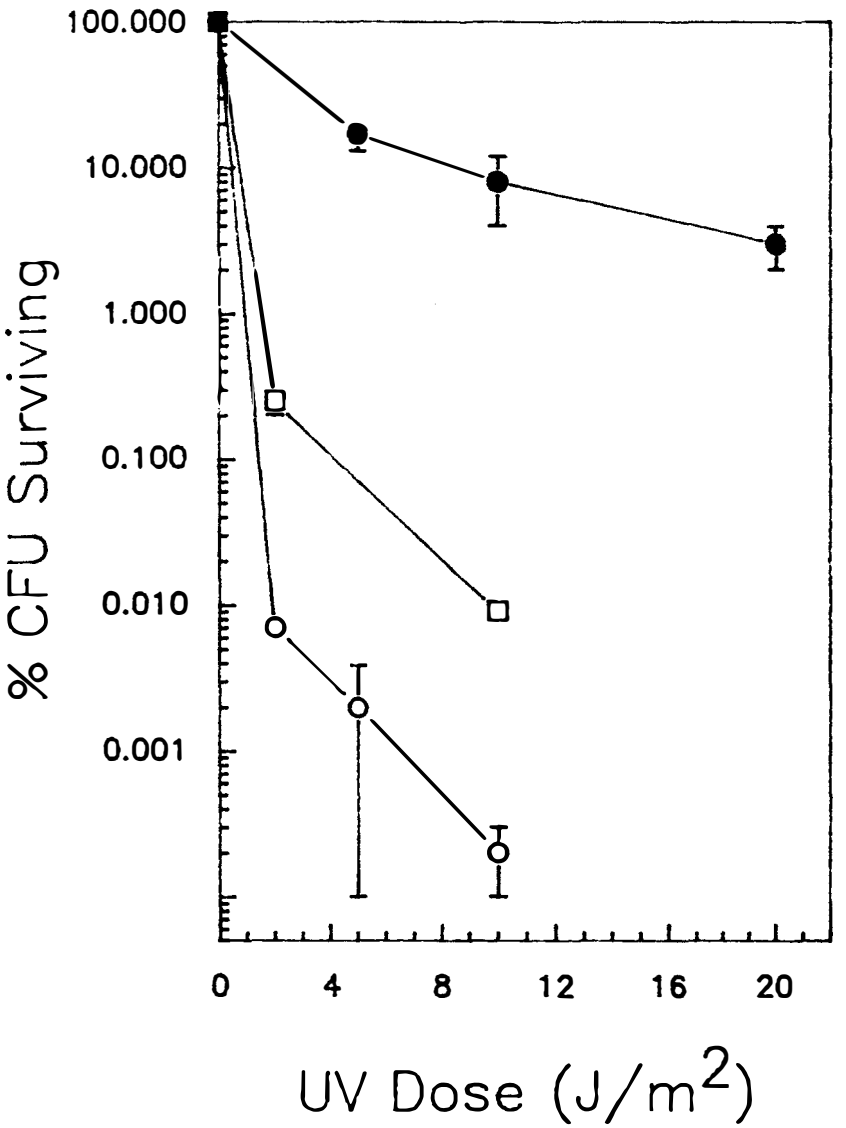

Tc r
pKML3001
23kb
S
Bg
cos
Bg
E
H
E
B

Figure 18. Restoration by pKML3001 to resistance to killing by UV irradiation of an *E. coli recA* mutant. Cells were grown to a density of approximately 10^8/ml in LB , pelleted, suspended in saline, and exposed to various fluences of UV irradiation. After appropriate dilution in saline, cells were plated in duplicate on L-agar and incubated at 37°C overnight in the dark. Mean values are plotted, range of data is indicated by bars. Experiments were performed at least twice. (o) *E. coli* HB101 (*recA13*); (●) *E. coli* RM2320 (*recA13*, pKML3001); (□) *E. coli* RM5002 (*recA13*, pCP13).


100.000
10.000
1.000
0.100
0.010
0.001
% CFU Surviving
0
4
8
12
16
20
UV Dose (J/m2)

aeruginosa strains using a triparental mating technique. Transconjugants were examined for suppression of the pleiotropic Rec$^-$ phenotype conferred by the rec-102 mutation.

UV sensitivity.

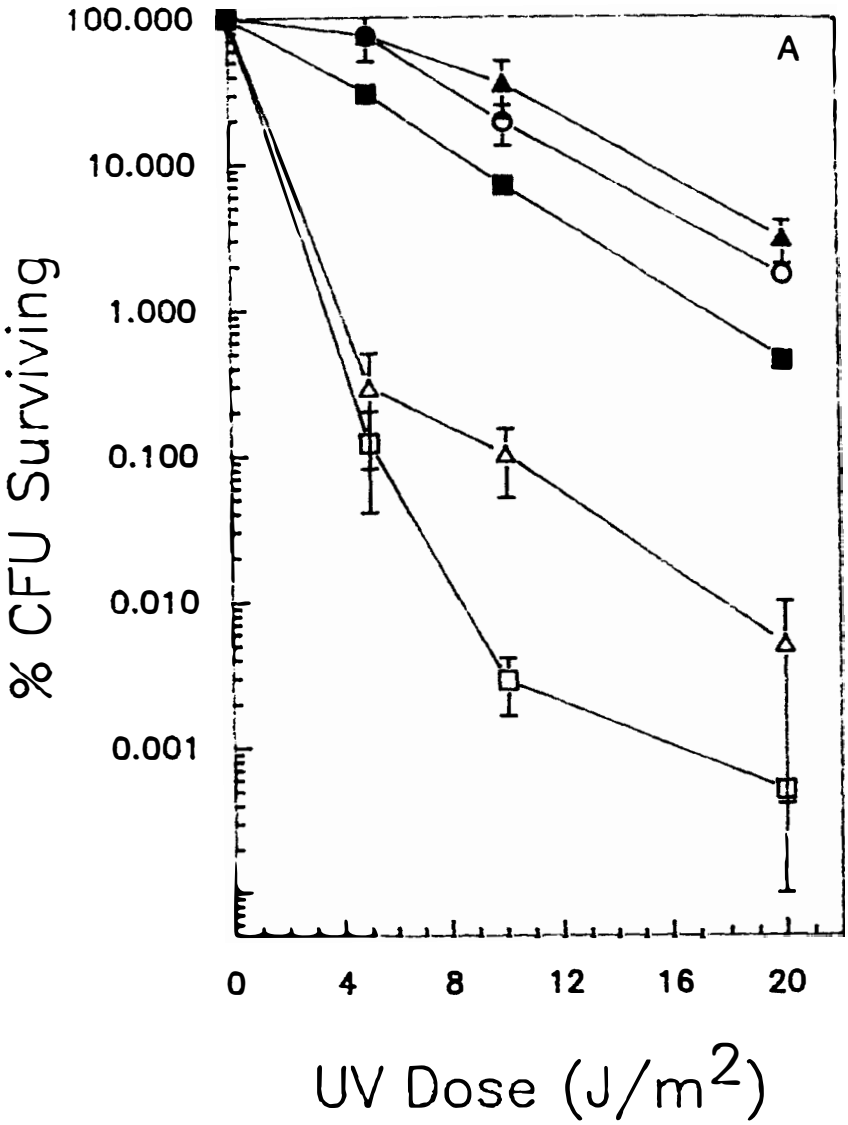
The sensitivity to UV irradiation of rec-102 mutant strains with and without pKML3001 was compared to PAO25, a Rec$^+$ strain of P. aeruginosa. The presence of the plasmid was found to confer greater resistance to UV irradiation upon the rec-102 mutants examined, with restoration to essentially wild-type levels in most, but not all, strains tested (Figure 19). In addition, pKML3001 was found to restore resistance of rec-102 mutants to MMS (data not shown).

Conjugational and recombinational proficiency.

The ability of rec-102 mutants containing pKML3001 to undergo homologous recombination after FP5-mediated conjugation was examined and compared to the efficiency of recombination in the absence of the plasmid (Table 13). The presence of the plasmid dramatically increased the number of recombinants recovered from Rec$^-$ recipient strains.

The ability of the various strains to acquire exogenous DNA through conjugation was assessed by determining the frequency of inheritance of the fertility

Figure 19. Restoration by pKML3001 to resistance to killing by UV irradiation of various rec-102 mutants of P. aeruginosa. Experiments were carried out as described in the legend to Figure 17. A representative experiment of at least two repetitions is shown. Mean values are plotted. Range of data is indicated by bars. (A): (△) P. aeruginosa PTO6003 (rec-102); (▲) P. aeruginosa RM2322 (rec-102, pKML3001); (□) P. aeruginosa PTO66 (rec-102); (■) P. aeruginosa RM2321 (rec-102, pKML3001); (o) P. aeruginosa PAO25 (Rec^+).


100.000
10.000
1.000
0.100
0.010
0.001
% CFU Surviving
A
0
4
8
12
16
20
UV Dose (J/m2)

(Figure 19, Continued)

(B): (○) PAO25 (Rec$^+$); (△) RM265 (rec-102); (▲) RM2323 (rec-102, pKML3001).

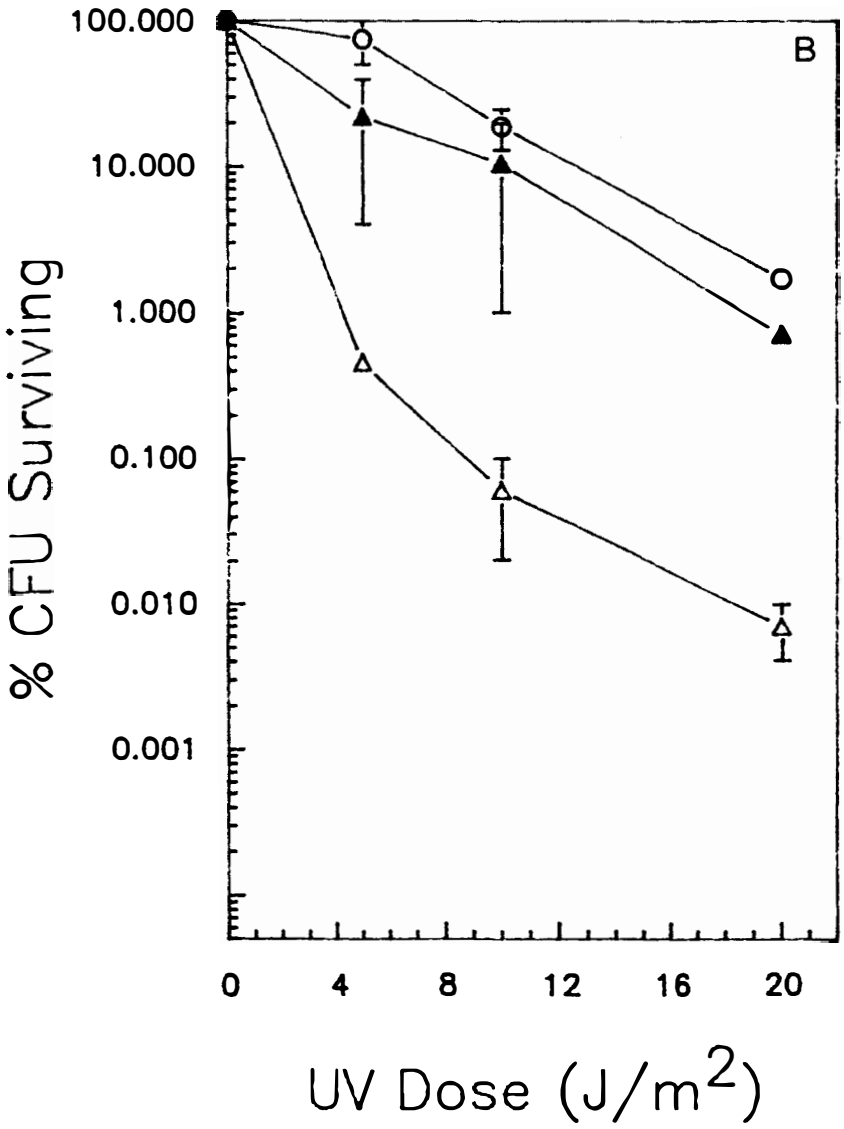

100.000
10.000
1.000
0.100
0.010
0.001
% CFU Surviving
B
0
4
8
12
16
20
UV Dose (J/m2)

Table 13: Recombinational and conjugal proficiency in FP5 crosses[a].

Recipient	Relevant Characteristics	Recombinational Proficiency[b] (Recombinants/100 Donors)		Plasmid Acquisition[c] (Hg^r Transconjugants/100 Donors)
PTO66	rec-102	(1)[d]	4.0×10^{-6}	1.6×10^{-1}
		(2)	$<3 \times 10^{-6}$	6.7×10^{-2}
RM2321	rec-102; pKML3001[e]	(1)	1.4×10^{-3}	5.0×10^{-1}
		(2)	1.0×10^{-3}	1.7×10^{-1}
PAO303	Rec^+	(1)	4.4×10^{-3}	2.4×10^{-2}

[a] RM187 was used as the donor in PTO66 matings and PAO832 in the PAO303 mating. Cells were mixed in a donor-to-recipient ratio of 1:1.2. Matings were performed for 2 h at 37°C in liquid medium.

[b] *his-4*$^+$ recombinants were selected in PTO66 matings and *argB21*$^+$ recombinants for the PAO303 mating.

[c] Transfer of FP5 was quantified by selection for Hg^r.

[d] Experiments were done twice except where indicated.

[e] pKML3001 contains the *P. aeruginosa recA* analogue.

plasmid FP5 (Table 13). Both Rec$^-$ and Rec$^+$ strains were able to receive and maintain FP5 at essentially the same levels.

Induction of prophage D3 from rec-102-containing strains.

The D3 prophage is inducible by UV irradiation in wild-type (Rec$^+$) lysogens of P. aeruginosa (61). In order to determine if UV induction of prophage D3 was dependent on functions encoded by plasmid pKML3001, the ability of various D3 lysogens of rec-102 mutants to release phage spontaneously and subsequent to UV irradiation was determined (Table 14). Lysogens of rec-102 strains were constructed by cross-streaking cells against a phage streak on L-agar. Survivors were tested for resistance to the cross-streaked phage and for spontaneous release of phage. While both rec-102-containing and wild-type strains are capable of spontaneously releasing D3 phage, the UV induction of prophage is inhibited by the presence of the rec-102 allele. Plasmid pKML3001 is capable of restoring the UV inducibility of D3 prophage from rec-102-containing lysogens.

Construction of a recA::Tn5 P. aeruginosa strain.

In an attempt to produce a Tn5-inactivated recA mutation in the chromosome of P. aeruginosa plasmid

Table 14: UV induction of D3 prophage.

Strain	Relevant Characteristics		Viability (CFU/ml)	Phage Produced (PFU/CFU)	
				Spontaneous	Induced[a]
RM247	Rec$^+$	(1)[b]	7×10^7	0.3	7.1
		(2)	7×10^7	0.3	7.1
RM276	rec-102	(1)	3×10^7	0.2	0.01
		(2)	3×10^7	0.1	0.07
RM2324	rec-102 pKML3001[c]	(1)	7×10^7	0.2	2.9
		(2)	7×10^7	0.6	4.3

[a]Cells were suspended in 0.85% saline and UV irradiated at a fluence of 10 J/m^2. The cells were suspended in Luria both and incubated 2 h at 37^o.

[b]Experiments were done two times. All platings were done in duplicate.

[c]pKML3001 contains the *P. aeruginosa recA* analogue.

pKML3001 was mobilized into the kanamycin supersensitive P. aeruginosa strain PAO4141. This plasmid is unable to replicate in P. aeruginosa so to produce kanamycin resistant cells, the Tn5 element must be retained by a homologous recombination event between the chromosome and appropriate DNA fragment of the plasmid. Alternatively, the Tn5 element may transpose to a new location. Kanamycin resistant clones were isolated and some did seem to be more sensitive to UV irradiation (Figure 20). Also this strain appeared to be recombinationally deficient in transduction with F116L (Table 15). Unfortunately, this phenotype was unstable with the continued growth of the strain.

Discussion

Examination of P. aeruginosa chromosomal DNA by the technique of Southern blotting has revealed that rec-102-containing strains contain a restriction endonuclease polymorphism near the region encoding the recA gene. This finding prompted a close examination of rec-102-containing strains to ascertain whether or not they could be complemented by the P. aeruginosa recA analogue. It is, however, impossible to determine if this actually

Figure 20. UV irradiation resistance of *recA*::Tn*5*-containing strains of *P. aeruginosa*. Experiments were performed as described in the legend to Figure 19. Mean values are plotted, range of data is indicated by bars. Experiments were performed at least twice. (●) PAO 4141 [Rec^+]; (○) RM9999 [*recA*::Tn*5*].

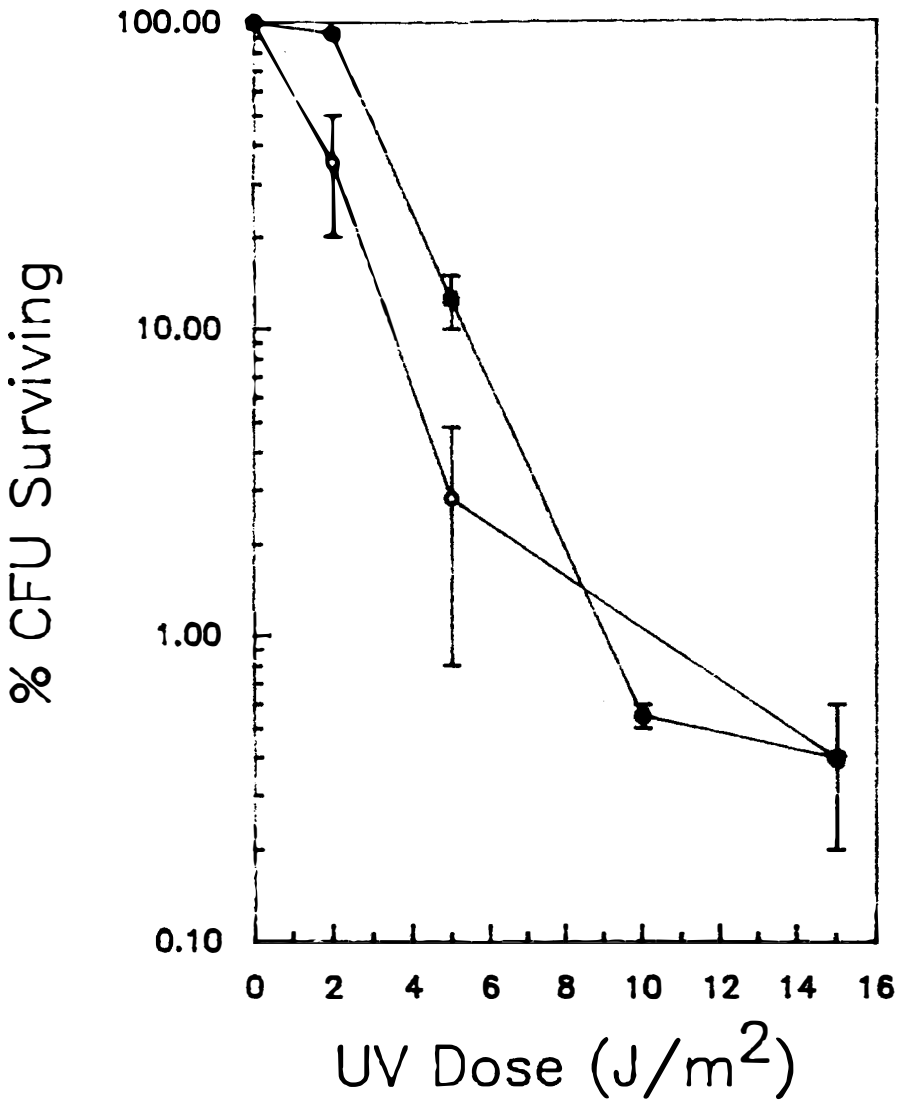

100.00
10.00
1.00
0.10
% CFU Surviving
0
2
4
6
8
10
12
14
16
UV Dose (J/m2)

Table 15. Recombinational proficiency following F116L transduction.

Strain	Relevant Characteristics		Recombinational Proficiency (Transductants/10^6 PFU)[a]
PAO4141	Rec^+	(1)[b]	3.0
		(2)	7.0
RM9999	*recA*::Tn*5*[c]	(1)	0.2
		(2)	0.2

[a] *met-9020*$^+$ transductants were selected. Phage were grown on PAO1.

[b] Experiments were done two times.

[c] The chromosomal *P. aeruginosa recA* was inactivated by homogenotization as described in the text.

represents a difference between P. aeruginosa strains PAO and PAT.

When a fragment of the P. aeruginosa PAO chromosome which is capable of complementing the activities of the RecA protein in various E. coli recA mutants (25) was subcloned into a broad-host-range vector and mobilized into several P. aeruginosa strains containing the rec-102 allele (43), the pleiotropic effects of this mutation were complemented by the cloned fragment.

The ability of the Rec^- strains to support homologous recombination with and without pKML3001 was examined (Table 13). The presence of the plasmid increased recombinational proficiency of rec-102 mutants approximately one-thousand fold in FP5- mediated crosses. A rough indication of the levels of recombinant formation possible in $RecA^+$ P. aeruginosa is given in Table 13 with the experiment using PAO303. While this strain may not be directly compared to the PTO strains, the marker selected (argB21) does map very near the his-4 marker and may be used as an approximate positive control since the strain representing the true positive control is not available.

Both strains with and without the plasmid are capable of receiving and maintaining plasmid DNA through conjugation at essentially the same level (Table 13).

There are some modest differences in the ability of these strains to receive DNA by conjugation, but not of sufficient magnitude to explain the differences in recombinant formation. In fact, the Rec^- strain receives DNA at a greater efficiency than Rec^+ strains. The defect in recombination in these Rec^- strains is, therefore, neither one of inability to receive DNA from donor cells, nor destruction of foreign DNA entering the cell. Therefore, the difference in levels of recombinant formation exhibited by these strains can be attributed to their relative proficiency at carrying out homologous recombination and not to any difference in conjugal ability.

The presence of a plasmid containing the P. aeruginosa recA analogue in rec-102 mutants confers in some cases greatly increased resistance to UV irradiation (Figure 19). In certain strains, pKML3001 restores UV resistance to the same level as wild-type P. aeruginosa strains. The difference in the ability of different P. aeruginosa strains to be complemented by plasmid pKML3001 is perplexing. Perhaps this reflects a difference in the recA gene itself produced during the construction of the mutant strains used for study. For example, if one strain inherited a mutated recA gene causing premature

translational termination of the protein product and another, due to intragenic recombination during construction procedures, received a gene encoding a missense protein, differences may be evident in the UV sensitivity of the strains. The production of a full-sized mutant protein could cause interference with wild-type RecA protein and make the cell containing genes specifying both proteins less able to be restored to wild-type UV resistance. In the other case cells containing genes with prematurely terminated or with promoter mutations that allow no protein production may not show such interference and may be complemented to wild-type levels.

Two characteristics associated with the SOS network in *E. coli* are the induction of error-prone DNA repair and the induction of prophage lambda by DNA damaging agents (111,141,153). Error-prone DNA repair is not induced by nalidixic acid (10) in either Rec^+ or Rec^- strains of *P. aeruginosa* PAO. However, the induction of D3 prophage is observed upon exposure of a Rec^+ D3 lysogen to UV irradiation. While the spontaneous release of phage is essentially the same in Rec^+ and Rec^- strains, the induction of D3 prophage by UV irradiation is dependent on the presence of a wild-type allele of *rec-102*. This phenotype is similar to that of *E. coli*

cells containing the recA142 allele (25,111). The presence of the cloned P. aeruginosa recA analogue within the cell allows the induction of D3 prophage by UV irradiation at levels equivalent to isogenic Rec^+ strains (Table 14). The UV induction of D3 prophage thus is dependent on the functions complemented by pKML3001. Preliminary experiments have indicated UV induction of P. aeruginosa prophage F116L is dependent on the functions complemented by pKML3001 (data not shown). This prophage induction provides the first clear demonstration that at least a subset of the DNA-damage inducible phenomena of E. coli occurs in P. aeruginosa and is dependent on the $RecA^+$ phenotype.

The P. aeruginosa RecA protein is capable of inducing prophages of three totally unrelated bacteriophages, D3 and F116 of P. aeruginosa and lambda of E. coli (data not shown). The repressors of phages D3 and F116L do not show sequence homology to each other or to the lambda cI repressor at least under the conditions of the test, that of one of high stringency. However, if the DNA sequence homology is less than 85%, no signal is observable under the experimental conditions used. The possibility still exists that the phages are somewhat homologous at the DNA sequence level with this homology

only detectable using hybridization and washing at lessened stringency. Possibibly limited convergent evolution of these temperate phages has occurred to take advantage of the potential of the RecA protein of their hosts to monitor the level of DNA damage to the cell and to cause prophage induction under appropriate conditions.

Attempts to construct a recA::Tn5 strain of P. aeruginosa were not successful. These strains were unstable and lost the Rec^- and UV^S phenotype spontaneously. These revertant strains did retain resistance to kanamycin and may have arisen from precise excision of Tn5 from the recA gene. Alternatively, the strains constructed may never have been true recombinants but, merodiploids of the recA region. Perhaps the presence of a Tn5-inactivated recA gene interfered with normal RecA protein function in a manner analogous to that described for certain truncated polypeptides in Chapter III. While the Tn5-inactivated gene did not have this effect in E. coli perhaps this is due to a more efficient expression of the gene in the P. aeruginosa background leading to a more efficient production of interfering polypeptides.

Based upon the data presented here, it may be concluded that the P. aeruginosa PAO chromosomal fragment which is contained in pKML3001 carries a gene whose

protein product carries out functions in P. aeruginosa analogous to the functions of the RecA protein of E. coli. Therefore, the P. aeruginosa PAO chromosomal fragment present in pKML3001 contains the P. aeruginosa recA gene. The data also supports the hypothesis that the rec-102 mutation is an allele of the P. aeruginosa recA gene. Unfortunately, the definitive demonstration of this hypothesis by marker exchange experiments was not possible due to the instability of the strains produced.

P. aeruginosa genes are usually only poorly expressed in E. coli (72), however, the recA analogue shows expression at levels adequate to allow complementation of recA E. coli mutants both in recombination and SOS induction phenomena (Chapters II and III). The recA gene, whatever its evolutionary origin, has clearly been functionally conserved.

CHAPTER V

ISOLATION OF THE PHAGE D3 c1 GENE

The establishment and maintenance of lysogeny by temperate bacteriophages requires the continued presence of specific repressor proteins (34,57). For phage lambda of E. coli these functions are supplied by the product of the cI gene (57,75,76). In E. coli the induction by DNA-damaging agents of the prophages of lambda and related viruses is initiated by the specific cleavage of the repressor of vegetative functions promoted by an activated form of the recA gene product (111,112,113,114). This cleavage takes place at a unique Ala-Gly bond within the repressor protein (111). The protein product of the recA gene of P.aeruginosa PAO is capable of mediating the induction by DNA-damaging agents of prophage lambda from recA mutants of E. coli as well as prophage D3 from recA mutants of P. aeruginosa (Chapters II, III and IV). The data suggest that the P.

aeruginosa RecA protein mediates this induction by a mechanism similar to that of the E. coli RecA protein (Chapter III).

D3 is a temperate bacteriophage of Pseudomonas aeruginosa which was originally described by Holloway et al. (62). The D3 virion is complex with a polyhedral head and a prominent tail with six knob-like projections (98). It contains a linear double-stranded DNA molecule of approximately 60 kb in size (98). The prophage integrates into the P. aeruginosa PAO genome (21) and is inducible to lytic growth by UV irradiation (62). This induction requires that the lysogenized host have a $RecA^+$ phenotype (Chapter IV) and leads to the formation of specialized transducing particles (21).

Egan and Holloway (39) demonstrated that the establishment and maintenance of lysogeny by phage D3 were dependent upon the expression of three genetic loci (c1, c2, and c3) within the D3 genome. Recently Gertman, et al. (46) have determined that the insertion of IS222 into a specific location in the D3 genome leads to the loss of the ability of the phage to establish lysogeny. They identified the location of this insertion by restriction endonuclease analysis.

The work in the preceeding Chapters has shown that phage D3 is UV inducible and that this induction requires

the RecA protein. In addition, certain Les$^-$ mutants of *P. aeruginosa* are phenotypically similar to *recA* mutants of *E. coli*. The Les$^-$ phenotype may be overcome by infecting the cell at elevated MOI (96) suggesting that destruction of a phage-encoded function, perhaps a repressor of vegetative functions, may be involved in the Les$^-$ phenotype. Taken together, the data indicate that RecA protein interacts with phage D3 under certain conditions and possibly an alteration in RecA protein activity leads to the Les$^-$ phenotype. In order to clarify the nature of the interaction of RecA protein and phage D3, the repressor of D3 vegetative function was cloned and analyzed. The designation *c1* has been used for this gene.

Materials and Methods

Bacteria, plasmids, and bacteriophage.

The bacterial strains used in this study are described in Table 16. The bacteriophage strains are listed in Table 17. pBR322 (13) and pME292 (70), a 6.8 kb *P. aeruginosa* plasmid derived from pVS1 (71) which is maintained at approximately 2 copies/chromosome in the cell were used as the cloning vectors. Cloning into the

Table 16. Bacterial strains.

Strain	Plasmid	Relevant genotype[a]												Other markers	Prophage[a]	Source or reference
		rec	arg	his	hsd	ilv	leu	lys	pro	pur	sup	trp	thi			
E. coli																
AB1157		+	E3	-4	+	+	B6	+	A2	+	E44	-1	-1			(26)
χ^{1488}		+	+	-53	R2	-277	+	C65	+	E41	+	+	-1	*minA1 minB2*		(27)
HB101		A13	+	+	S20	+	-	+	A2	+	E44	+	-1			(13)
RM1154	pBR322	+	E3	-4	+	+	B6	+	A2	+	E44	-1	-1			AB1157
RM1157	pKML11	+	E3	-4	+	+	B6	+	A2	+	E44	-1	-1			AB1157
RM1158	pKML12	+	E3	-4	+	+	B6	+	A2	+	E44	-1	-1			AB1157
RM1160	pBR322	+	E3	-4	+	+	B6	+	A2	+	E44	-1	-1		(λ R)	RM1154
RM1163	pKML11	+	E3	-4	+	+	B6	+	A2	+	E44	-1	-1		(λ R)	RM1157
RM1164	pKML12	+	E3	-4	+	+	B6	+	A2	+	E44	-1	-1		(λ R)	RM1158
RM1184		+	E3	-4	+	+	B6	+	A2	+	E44	-1	-1		(λ R)	AB1157
RM2330	pKML1101	+	E3	-4	+	+	B6	+	A2	+	E44	-1	-1			AB1157

(Table 16: Continued)

Strain	Plasmid	Relevant genotype[a]												Other markers	Prophage[a]	Source or reference
		rec	arg	his	hsd	ilv	leu	met	pro	pur	sup	thr	thi			
RM2331	pKML11	+	+	-53	R2	-277	+	C65	+	E41	+	+	-1	minA1 minB2		X^{1488}
RM2332	pKML1101	+	+	-53	R2	-277	+	C65	+	E41	+	+	-1	minA1 minB2		X^{1488}
P. aeruginosa																
PAO1		+	+	+	+	+	+	+	+	+	+	+	+			(96)
PAO25		+	F10	+	+	+	-10	+	+	+	+	+	+			(43)
PAO38		+	+	+	+	+	-38	+	+	+	+	+	+			(43)
RM17	FP2	+	+	+	+	+	-38	+	+	+	+	+	+		(D3)	PAO38
RM247		+	F10	+	+	+	-10	+	+	+	+	+	+		(D3)	PAO25
RM2130	pME294	+	F10	+	+	+	-10	+	+	+	+	+	+			PAO25
RM2131	pKML5	+	F10	+	+	+	-10	+	+	+	+	+	+			PAO25
RM2132	pKML6	+	F10	+	+	+	-10	+	+	+	+	+	+			PAO25
RM2327	pKML7	+	F10	+	+	+	-10	+	+	+	+	+	+			PAO25
RM2328	pKML8	+	F10	+	+	+	-10	+	+	+	+	+	+			PAO25

(Table 16: Continued)

Strain	Plasmid	Relevant genotype[a]												Other markers	Prophage[a]	Source or reference
		rec	arg	his	hsd	ilv	leu	met	pro	pur	sup	thr	thi			
RM2333	pKML6	+	F10	+	+	+	-10	+	+	+	+	+	+		(D3)	RM2131
RM2334	pME294	+	F10	+	+	+	-10	+	+	+	+	+	+		(D3)	RM2130
RM2335	pME294	+	F10	+	+	+	-10	+	+	+	+	+	+		(F116L)	RM2130
RM2336	pKML6	+	F10	+	+	+	-10	+	+	+	+	+	+		(F116L)	RM2131
RM2337		+	F10	+	+	+	-10	+	+	+	+	+	+		(F116L)	PA025

[a]Genotype symbols and abbreviations are as specified by Bachmann (3) except fon which indicates resistance to phage F116L. Prophage are symbolized by including the name of the phage in parenthesis when present.

Table 17. Bacteriophage.

Strain	Relevant genotype	Source or reference
Phage Lambda		
λR	*R5am*	R. E. Malone
mms813	*vir*	F. W. Stahl
λ207	*cI ind*	F. W. Stahl
λ*imm*^{434}	*imm*^{434}	M. Casadaban
JMC307	*b1453 J6am cI857*	F. W. Stahl
Pseudomonas Phage		
D3	wild-type	(62,98)
D3c	*c1-3*	Spontaneous clear-plaque mutant of D3
F116L	wild-type	(62,98)

unique HindIII site of pME292 inactivates the kanamycin resistance locus and selection for the plasmid is made by carbenicillin resistance (Cb^r) (69). pME294 is identical to pME292 except that it is carried in the cell at approximately 15 copies/chromosome. pKML5, pKML6, pKML7, and pKML8 are clones of the D3clrR gene in pME292, and pKML11 and pKML12 are clones of clrR in pBR322. pKML1101 is a deletion derivative of pKML11 which inactivates the c1 gene. These recombinant plasmids are described below in detail.

Media and culture conditions.

Bacteria were maintained on LB or L-agar (Chapter II). Plasmid containing cells were selected using 50 ug ampicillin (Ap)/ml of medium for E. coli and 500 ug Cb/ml of medium for P. aeruginosa. Cells were grown in M9 medium (Chapter III) for minicell analysis of the protein products of plasmid encoded genes. TM, TMN, TE and BSG buffers have been described (Chapters II and III). All restriction enzymes were used as described in Chapter II.

Lysates of bacteriophage lambda were prepared as described in Chapter II. Lysates of bacteriophage D3 were prepared as described in Chapter IV.

DNA isolation.

D3 DNA was isolated from the virion by the method of Hinkle and Miller (58). Phage lysates, prepared as

described in Chapter IV, were pelleted by centrifugation at 25,000 rpm for 60 min in a Beckman L5-65 centrifuge using a Ti50 rotor. The pelllet was suspended in one ml lambda buffer [0.2% (w/v) KH_2PO_4, 0.7% (w/v) K_2HPO_4 and 0.0025% (w/v) $MgSO_4$ $7H_2O$, pH 7]. The pellet was allowed to stand at room temperature to aid resuspension which was done gently. Sarkosyl was added to a final concentration of 5% (w/v) and the suspension heated to 60°C for 10 min. An equal volume of lambda buffer-saturated phenol was added and the solution extracted. The DNA-containing (aqueous) phase was centrifuged and aqueous phase retained. The aqueous phase recovered was dialysed against four liters of TE buffer at 4°C.

Plasmid DNA was isolated by the the alkaline lysis method for large-scale isolation of plasmid DNA as described by Maniatis, et al. (86). A 500 ml culture of cells was harvested by centrifugation and suspended in 10 ml of 50 mM glucose, 10 mM EDTA, 25 mM Tris (pH 8) and 5 mg lysozyme/ml solution. The solution was allowed to stand at room temperature for five min. To lyse the cells, 20 ml of a solution of 0.2 N NaOH, 1% (w/v) SDS was added and the mixture incubated on ice 10 min. At the conclusion of this incubation, 15 ml of the potassium acetate solution described in Chapter II for rapid clone

analysis was added and the solution incubated 10 min on ice. The solution was centrifuged at 20,000 rpm in a Sorvall RC5-B centrifuge in an SS-34 rotor for 20 min at $4^{o}C$. The supernatant was retained and 0.6 volume of ispropanol added. The solution was incubated at room temperature for 15 min and centrifuged in a Sorvall RC5-B 12,000 rpm in the SS-34 rotor for 30 min at room temperature. The pellet was retained and suspended in eight ml TE buffer.

CsCl-ethidium bromide density equilibrium purification of the DNA was carried out in a Beckman TL-100 ultracentrifuge at 100,000 rpm using a TLA 100.2 fixed angle rotor following the procedure described in Chapter II.

Cloning of the D3 c1 gene.

Phage D3 DNA (0.25 ug) isolated from the virion was digested to completion with HindIII (nine fragments), ethanol precipitated, and suspended in TE buffer. This phage DNA was mixed with CsCl purified, HindIII-cleaved pME292 DNA which had been dephosphorylated with calf intestinal alkaline phosphatase (Chapter II). Vector and insert were suspended at a concentration of 10 ug/ml in ligation buffer (Chapter II), ATP (0.5 mM) and T4 DNA ligase (0.1 Weiss unit) were added and ligation was carried out at $16^{o}C$ for 2 h. The ligated DNA was used to

transform PAO25 using the method of Mercer and Loutit (92). Cells were grown to approximately 60 $Klett_{660}$ units in LB at 37°C. The cells were harvested by centrifugation at 5,000 x g and suspended in one-half their original volume in 150 mM $MgCl_2$. The cells were incubated at 0°C for 30 min and harvested by centrifugation as above taking care to keep them chilled. The cells were suspended in one tenth their original volume in 150 mM $MgCl_2$ and incubated as above for 30 min. DNA was added and incubation continued for 20-60 min. The cells were heat shocked by incubating at 37°C for two min. One ml LB was added and the cells incubated for 60 min to allow expression of plasmid genes. The cells were spread on LA supplemented with the appropriate antibiotic to select transformed cells.

Two hundred independent Cb^r clones were isolated and screened for their resistance to infection by a clear-plaque mutant of D3. Resistant clones were purified and plasmid DNA was isolated from them by a cleared lysate technique (7). This DNA was used to prepare a restriction map of the clone and to transform various strains of P. aeruginosa.

The insert from one of these recombinant plasmids (pKML6) was cloned into pBR322 to allow introduction of

the cloned D3 fragment into *E. coli* strain HB101 by the method of Davis *et al.* (33). Selection was made for Ap^r clones. The presence of the insertion fragment was confirmed by restriction endonuclease analysis of DNA prepared by the rapid clone analysis method of Maniatis, *et al.* (86). Clones with the D3 DNA inserted in opposite orientations were selected and designated pKML11 and pKML12.

Preparation of lysogenic strains.

Lysogens were constructed by cross-streaking the bacterial strain perpendicular to a cross streak of phage lysate on L-agar. The bacterial strain grows normally until phage is contacted where a zone of cell killing by the phage is noted. Cells from this region were streaked for isolation on L-agar and patched onto a freshly seeded lawn of phage-sensitive bacteria. The growth of a lysogen will result in spontaneous phage release and a zone of lysis of sensitive cells surrounding the patch. The immunity properties of lysogens were determined by cross-streaking the strain against stocks of a clear plaque mutant of the phage. Strains lysogenized by, or resistant to, a phage will show no killing at the intersection of the streaks.

Efficiency of plating.

The efficiency of plating of phages for different

bacterial strains was determined by serial dilution of the phage and plating on the strain to be tested and the reference strain (PAO303). The number of PFU resulting on the reference strain was defined as unity (1.00).

Induction of prophage.

Prophages of D3 and F116L were induced by UV irradiation as described in Chapter IV. Various exposures to UV were used as described below. Lambda prophage were induced with MMC as described in Chapter II.

Minicell analysis of pKML11.

Plasmids to be analyzed were introduced in the minicell-producing strain E. coli $_{X}$1488. Minicells were prepared as described in Chapter III. Labeling was carried out using [^{35}S] methionine (specific activity 1114 Ci/mmole; New England Nuclear). Labeling procedure and protein analysis using SDS polyacrylamide-gel electrophoresis and autoradiography were carried out as described in Chapter III.

Southern analysis.

D3, lambda, pKML6, and pKML11 DNAs were purified as described above, digested with HindIII or BamHI, electrophoresed in 0.7% agarose gels, and blotted onto nitrocellulose using capillary transfer as described in

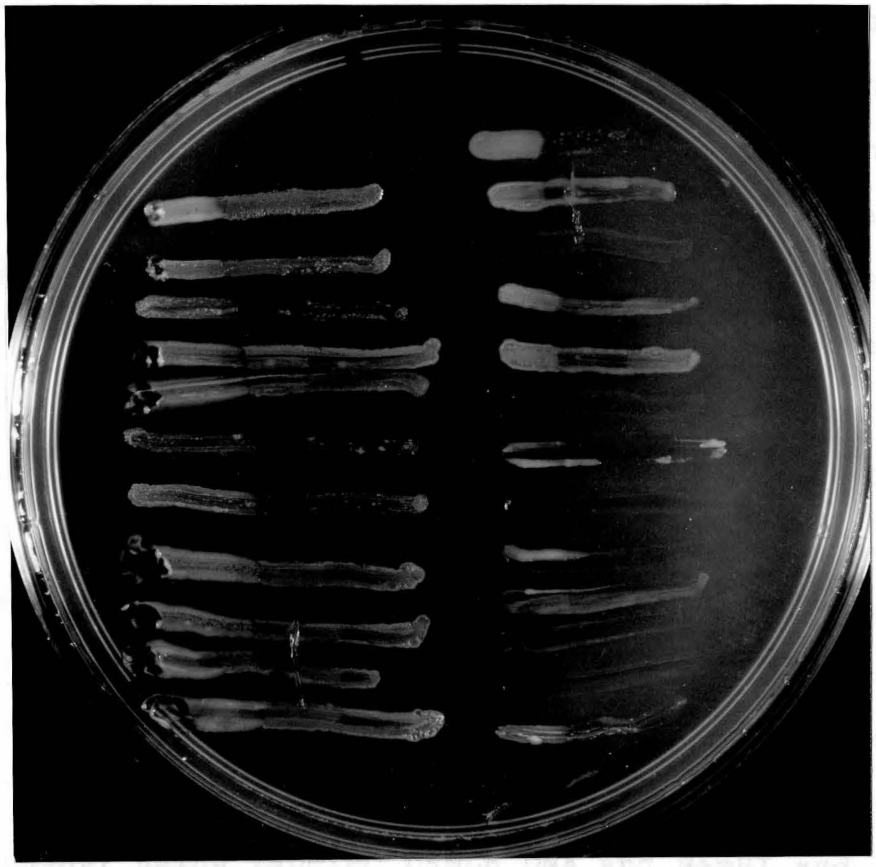
Chapter III. Purified pKML6 and pKML11 DNAs were labeled with [-^{32}P]-dCTP (800 Ci/mmole; New England Nuclear) by nick translation using a kit obtained from Amersham Corp. Unincorporated nucleotides were removed by the spun column procedure of Maniatis, et al. (86). These plasmid DNAs were used to probe the nitrocellulose blots of phage and plasmid DNAs by Southern analysis under conditions of high stringency as described by Maniatis, et al. (86).

Results

Cloning of the D3 c1 gene.

A HindIII digest of phage D3 DNA was inserted into pME292 and used to transform P. aeruginosa PAO25 as described above. Isolated clones were cross-streaked (96) against a lysate of D3c, a putative c1 repressor mutant, (10^9 PFU/ml) and clones which showed pseudoimmunity to infection by this clear-plaque mutant were utilized for further study (Figure 21). Plasmid DNA from several independent clones was isolated and a portion of each sample digested with HindIII. Each clone was shown to contain the same 9 kb insert. The remainder of the DNA from these preparations was used to transform PAO25. Greater than 99% of the transformants generated were pseudoimmune as judged by cross-streaking

Figure 21. Selection of potential D3 c1 clones. Transformants were cross streaked against D3c. Parental strain is at upper right. Those clones showing increased immunity to D3c were selected for further evaluation.



against D3c. The efficiency of plating of D3c and the heteroimmune phage F116L on the various clones was compared to PAO25 and several D3 lysogens (Table 18). The presence of the cloned D3 fragment in PAO25 reduced the plating efficiency of D3c >500 fold, to a level comparable to that observed with a true D3 lysogen. The D3c plaques observed on the strains containing the cloned fragment were extremely small and very turbid indicating that the cloned fragment could suppress the clear-plaque phenotype of D3c. The clone had no effect on the plating efficiency of F116L. The construction contained in RM2132 (pKML6) was chosen for further study.

A restriction map of the insert in pKML6 was prepared (Figure 22). This map indicates the cloned DNA fragment in pKML6 is derived from the same region of the phage genome determined by Gertman, et al. (46) to contain genes necessary for turbid plaque formation. Southern hybridization analysis under conditions of high stringency using labeled pKML6 DNA and BamHI and HindIII digests of D3 DNA yielded the predicted patterns of homology (data not shown). There was no homology to phage lambda or F116L DNA detected in this analysis.

Induction of D3 prophage in the presence of pKML6.

The induction of D3 prophage by UV irradiation is dependent on a RecA$^+$ phenotype (Chapter IV). If the

Table 18. Effects of the cloned D3 c1 gene on the efficiency of plating.

Strain	Plasmid	Prophage	Relative EOP[a]	
			D3c	F116L
PAO25	---[b]	---	1.0[c]	1.0
RM247	---	D3	2×10^{-2}	1.0
RM17	---	D3	2×10^{-3}	1.0
RM2130	pME294	---	0.7	1.0
RM2131	pKML5[d]	---	1×10^{-2}	ND[c]
RM2132	pKML6	---	2×10^{-3}	0.8
RM2327	pKML7	---	2×10^{-3}	ND
RM2328	pKML8	---	2×10^{-3}	ND

[a]EOP of strain relative to EOP of strain PAO25

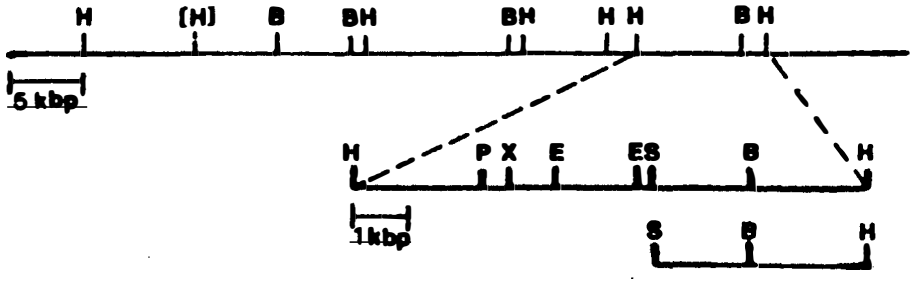
[b]None present.

[c]Experiments were done three times. Average values are reported. The independent determinations did not vary from the mean by more than 20%.

[d]pKML5, pKML6, pKML7, pKML8 are independent clones of the D3 c1 gene in pME292.

[c]Not done.

Figure 22. Restriction endonuclease map of phage D3. pKML6 contains the c1 gene. pKML1101 is a SalI deletion which inactivates the c1 gene.



D3

pKML6

pKML1101

H-*Hind*III

B-*Bam*HI

S-*Sal*I

E- *Eco*RI

P- *Pst*I

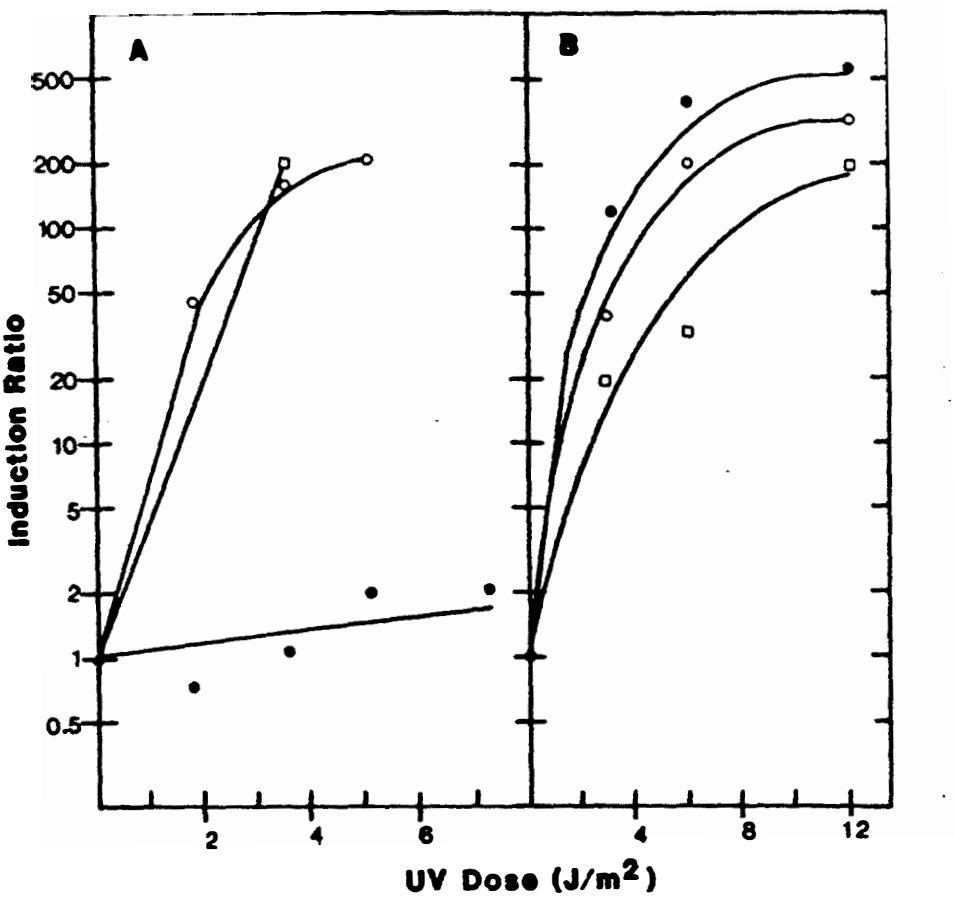
X-*Xho*I

mechanism of induction of prophage D3 is the destruction of the c1 repressor upon exposure to a DNA damaging agent, it should be possible to decrease this effect by increasing the concentration of D3 c1 repressor in the cell. Such a case has been shown to be true for lambda prophage in E. coli (6,106,111). This interference with prophage induction by overproduction of repressor has been termed subinduction. When the cloned lambda cI is introduced into a lambda lysogen, the increased concentration of repressor protein produces conditions in which the prophage is not induced upon exposure to an inducing agent. Increasing the dosage of the D3 repressor gene by introducing pKML6 into the lysogenic strain should therefore inhibit induction of the D3 prophage by UV irradiation. Isolates of RM247 were prepared containing pKML6 (RM2335) and pME294 (RM2334). The ability of UV irradiation to induce lytic growth of the resident prophage in each of these strains was analyzed (Figure 23). The presence of pKML6 specifically inhibited the induction of the D3 prophage by UV irradiation. The induction of the heteroimmune prophage F116L was not inhibited by the presence of pKML6 (Figure 23).

Identification of the c1 gene product.

The D3 DNA insert in pKML6 was subcloned into the

Figure 23. UV induction of prophage. Induction experiments were carried out using UV irradiation. Induction ratio is number of pfu/ml at time t divided by number of pfu/ml at time of induction. Average values of two repetitions are shown. Range of induction ratios did not vary by more than 20%. (A) D3 lysogens: (○) RM247 (PAO25 [D3]); (●) RM2333 (pKML6); (□) RM2334 (pME294). (B) F116L lysogens: (□) RM2335 (pME294); (●) RM2336 (pKML6); (○) RM2337 (PAO25 [F116L]).


A
B
500
200
100
50
20
10
5
2
1
0.5
Induction Ratio
2
4
6
4
8
12
UV Dose (J/m2)

HindIII site of pBR322 and introduced into E. coli HB101. Plasmids were selected with the insert in opposite orientations with respect to the Tetracycline resistance promoter (104). They were designated pKML11 and pKML12 (Figure 24). A deletion subclone of pKML11, pKML1101, was prepared by cleavage of CsCl purified pKML11 DNA with SalI and re-ligation (Figure 24).

pKML11 and pKML1101 were introduced into the minicell producing strain E. coli $_{X}$1488. Minicells were prepared, the plasmid-encoded proteins labeled with [^{35}S]-methionine, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (Figure 25). pKML11 produced a protein of 37,000 MW which was not encoded by pKML1101.

Effects of pKML11 and pKML12 on the induction of lambda prophage in E. coli.

pKML11 and pKML12 were introduced into lambda lysogens of E. coli AB1157 and the induction of the prophage attempted using MMC. The presence of pKML11 inhibited the induction of the lambda prophage while the presence of pKML12 and pBR322 had no effect on this process (Table 19).

EOP of phage lambda on strains containing pKML11 and pKML12.

If the D3 c1 gene product were acting

Figure 24. Derivation of pKML11, pKML12, and pKML1101. Restriction enconuclease symbols are: B, BamHI; C, ClaI; E, EcoRI; H, HindIII; P, PstI; S, SalI; and X, XhoI. Dots represent promoter regions of plasmid genes.

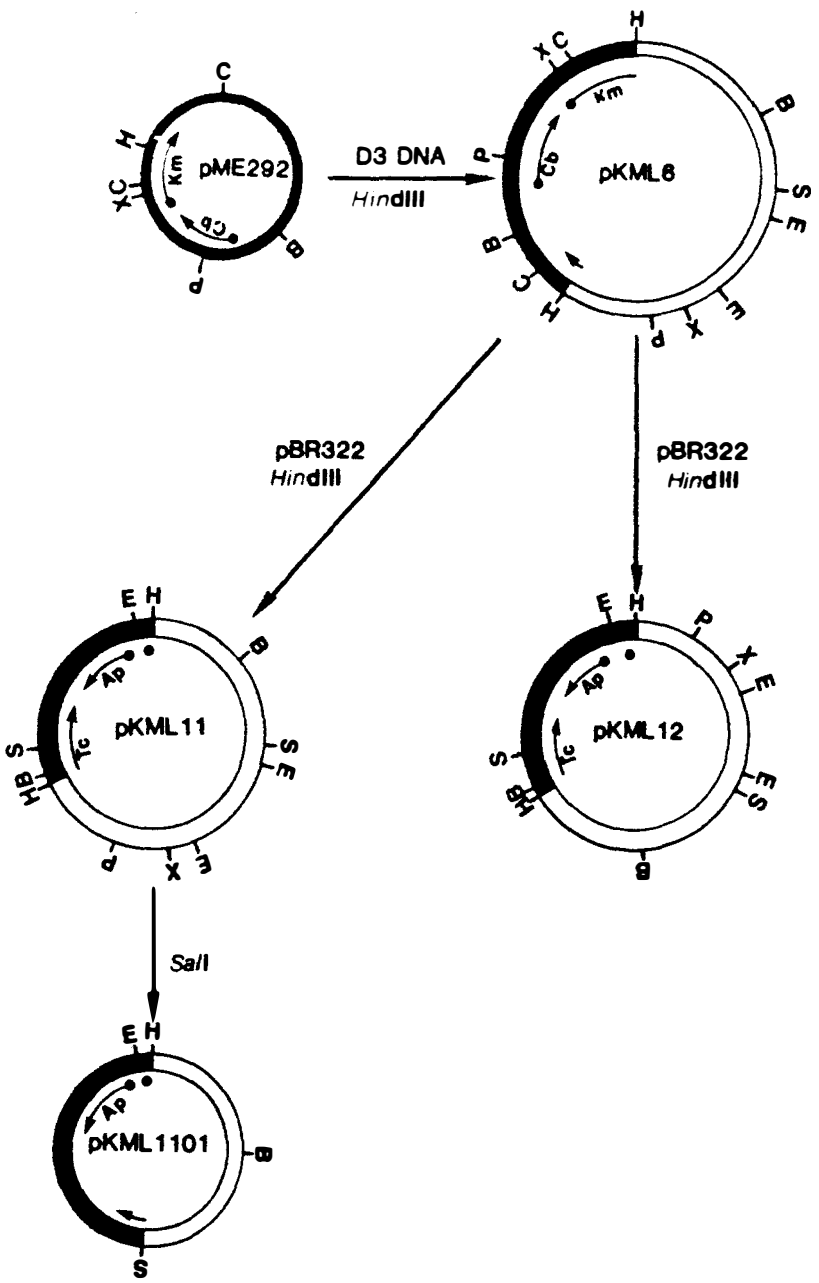

pME292
D3 DNA
HindIII
pKML6
pBR322
HindIII
pBR322
HindIII
pKML11
pKML12
SalI
pKML1101

Figure 25. Identification of proteins encoded by pKML11 and pKML1101. [^{35}S]-labeling of plasmid encoded proteins was carried out as described in the text. (A) RM2332 (pKML1011); (B) RM2331 (pKML11). Migration of strandard molecular weight markers is indicated to the left. Arrow indicates protein unique to c1-complementing clones. Plasmid pKML11 contains the c1 repressor, pKML1101 does not express repressor activity.

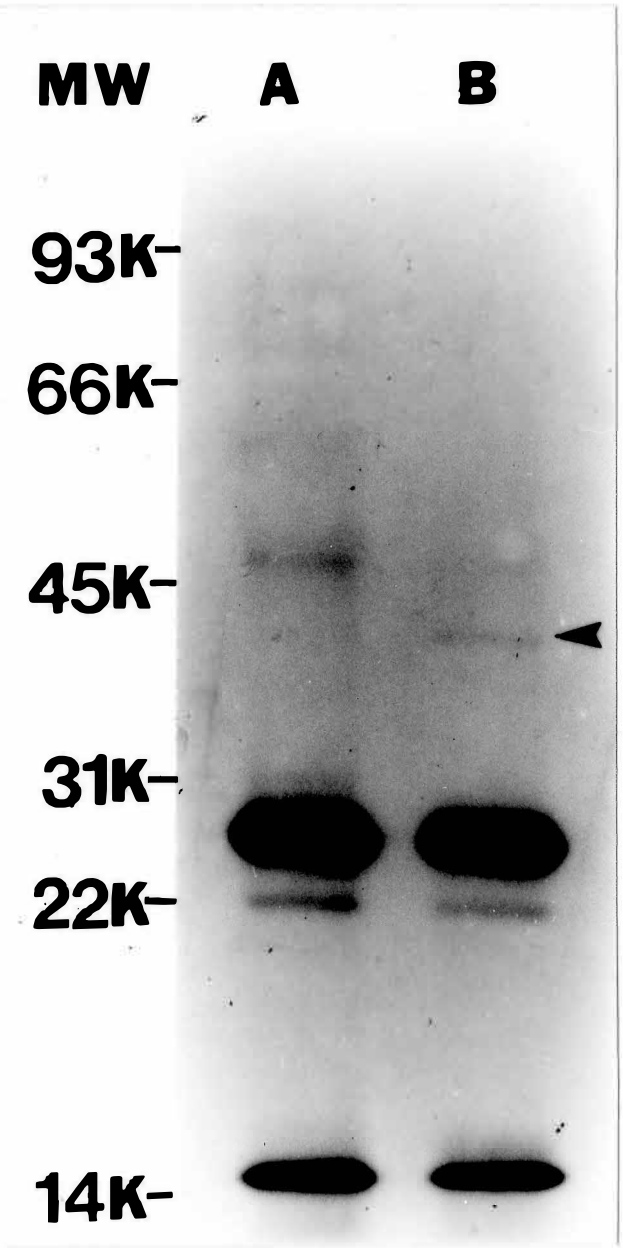

MW
A
B
93K-
66K-
45K-
31K-
22K-
14K-

Table 19. Effect of the cloned D3 c1 gene on the induction of λR prophage by mitomycin C.

Strain	Plasmid		Viability (CFU/ml)	Phage Produced (PFU/10^4 CFU)	
				Spontaneous	Induced[a]
RM1184	---[b]	(1)[c]	6×10^8	1.5	26,000
		(2)	6×10^8	2.0	31,000
RM1160	pBR322	(1)	4×10^8	1.0	4,750
		(2)	4×10^8	2.0	6,000
RM1163	pKML11[d]	(1)	8×10^8	6.2	18
		(2)	8×10^8	2.5	20
RM1164	pKML12	(1)	4×10^8	0.2	500
		(2)	4×10^8	0.3	1,750

[a]Cells were incubated with mitomycin C at 5 ug/ml for 150 min.

[b]None present.

[c]Experiments were done two times. Platings were done in duplicate.

[d]pKML11 and pKML12 contain the D3c1 gene cloned into pBR322 in opposite orientations.

nonspecifically in inhibiting the induction of lambda lysogens by simply titrating activated E. coli RecA protein, the ability of phage lambda to infect and grow lytically in strains of E. coli containing clones of the D3 c1 gene would not be affected. When this was tested (Table 20), it was found that the EOP of wild-type lambda on strains containing pKML11 was dramatically reduced. This inhibition appears to be specific for lambda as both virulent (repressor operator) mutants of lambda and imm^{434} phage are not affected by the presence of the plasmid. A mutant carrying the temperature sensitive mutation cI857 was inhibited at both the permissive and non-permissive temperatures. Neither pKML1101, the deletion derivative of pKML11, nor pKML12 had an inhibitory effect on the EOP of lambda.

Discussion

A 9 kb DNA fragment of the D3 genome has been isolated which complements D3c mutants to allow turbid plaque formation. The gene responsible for this complementation has been designated c1. The product of this gene represses lytic functions of the phage and the cloned fragment imparts immunity to superinfection by phage D3 to cells containing it. The product of the c1

Table 20. Effect of the cloned D3 cI gene on the efficiency of plating of phage lambda.

Bacterial Strain	Plasmid	Prophage	Relative EOP of Phage lambda[a]					
			Phage strain					
			λ R (cI$^+$)	λ JMC307 (cI857)		λ mms813 (vir)	λ 207 (ind)	λ imm^{434} (imm^{434})
				30°C	43°C			
AB1157	—[b]	—	1.00	1.00	1.00	1.00	1.00	1.00
RM1184	—	λ R	<8 x 10^{-6}	<2 x 10^{-5}	<2 x 10^{-5}	1.00	<5 x 10^{-6}	ND[c]
RM1154	pBR322	—	1.00	1.00	1.00	1.00	0.44	ND
RM1157	pKML11	—	<8 x 10^{-6}	<2 x 10^{-5}	<2 x 10^{-5}	1.00	<5 x 10^{-6}	1.00
RM1158	pKML12	—	0.25	ND	ND	1.00	1.00	ND
RM2330	pKML1101	—	1.00	ND	ND	1.00	1.00	ND

Footnotes to Table 20:

[a] EOP of bacterial strain relative to EOP of AB1157 for the same strain of phage. All experiments were done 3 times except for λR and λ207 which were done 6 times. Average values are reported. Independent determinations did not vary from the mean by more than 20%. Phage titers on AB1157 were 1×10^9 except for JMC307 which was 1×10^8.

[b] None present.

[c] Not done.

gene has been tentatively identified as a protein of approximately 36,000 molecular weight. Plasmid pKML11 produces several proteins. The presence of this 36,000 dalton polypeptide is correlated with the effects upon lambda phage infection. Expression of the gene product of the cloned c1 gene in E. coli is dependent upon the orientation of the inserted D3 DNA fragment in pBR322. pKML11 apparently expresses the gene more efficiently than pKML12 (Table 19). Plasmid pKML12 does seem to expresss the c1 function in E. coli and cause some reduction in spontaneous and induced levels of phage. The inserts in pKML6 and pKML11 both have the same orientation with respect to nearby external promoters normally utilized in their respective vectors for expression of drug resistance (13,69,104). This suggests that the D3 c1 gene may be utilizing an external promoter for expression and, therefore, may not be subject to the same regulation of expression as it is when part of the intact D3 genome. Whether or not the c1 gene is expressed as a part of a polygenic messenger is unknown. However, it is possible that transcription originating at the plasmid drug resistance promoters continues into D3 DNA and is responsible for a more efficient expression of these genes in E. coli.

D3 lysogens are inducible by UV irradiation in Rec$^+$ strains of P. aeruginosa (61,62). Mutations in the P. aeruginosa recA gene eliminate this induction (Chapter IV). The P. aeruginosa recA gene product is also capable of allowing induction of lambda prophage from recA mutants of E. coli (Chapters II and III). In E. coli the induction of lambda prophage after exposure to DNA damaging agents is known to be the result of the specific cleavage of the lambda cI repressor promoted by an activated form of the RecA protein (111). The data available to date suggest that the P. aeruginosa RecA protein may be responsible for the induction of D3 prophage by a similar mechanism.

In E. coli, overproduction of a particular immunity-type repressor inhibits the induction of resident prophage of that specific immunity group only (6,28,111). Increasing the concentration of cI repressor by addition to the cell of the cloned cI gene does not saturate activated RecA protein since prophage of other distinct immunity groups which are present are induced normally (6). Instead, it appears that the increased concentration of cI protein shifts the equilibrium between the monomeric and dimeric forms of the repressor to favor increased concentrations of dimer which is less susceptible to cleavage by the activated RecA protein

(106). Thus, inactivation of the overproduced cI repressor does take place upon exposure to an inducing treatment, but the decrease in effective repressor concentration is such that the lambda prophage is induced suboptimally. This phenomenon has been termed subinduction (6). D3 lysogens of P. aeruginosa harboring pKML6 are likewise incapable of inducing the resident prophage after exposure to UV irradiation. The presence of the D3 c1 clone in a D3 lysogen of P. aeruginosa may inhibit induction by a mechanism analogous to that of subinduction of phage lambda. Consistent with this hypothesis is the observation that lysogens of the heteroimmune phage F116L are induced normally in cells containing pKML6.

Phages D3 of P. aeruginosa and lambda of E. coli have several characteristics in common. They are both specialized transducing phages (21). Both appear to exercise a choice between lytic or temperate growth subsequent to infection of the cell, and their prophages integrate into unique sites in their respective hosts' chromosomes (21). However, their receptors are different and lambda will not infect P. aeruginosa nor will D3 infect E. coli (data not shown). While both are complex icosohedral phages, the morphology of the two phage

virions is different (2,98). Their genomes, while sharing a similar GC content, have unique restriction maps, are quite different in size (2,91), and Southern hybridization, under conditions of high stringency, has revealed no large regions of DNA sequence homology (data not shown). However, it is possible that the DNA sequences of the two phages are homologous at a lower level and that washing the blots under conditions of lesser stringency would reveal DNA base sequence homology.

The introduction of the cloned D3 c1 gene into an E. coli lambda lysogen caused the inhibition of prophage induction by mitomycin C. This observation suggests that the D3 repressor protein acts in some fashion to protect the lambda cI protein from destruction by activated RecA. If the present model of the subinduction phenomenon is correct, it seems most plausible that the D3 c1 repressor inhibits lambda prophage induction directly, perhaps by interacting with lambda cI protein to form heterodimers of the two phage repressors, thereby increasing the effective concentration of repressor dimers in the cell which are less susceptible to inactivation by activated E. coli RecA protein. Alternatively, the D3 c1 gene product could exert protection by directly inhibiting the activation of RecA protein. While certain plasmids have

been observed to inhibit the induction-promoting activity of the recA441 allele (4,5), it seems unlikely that the D3 c1 protein acts by this mechanism since the D3 prophage is itself UV inducible. Such an effect would be expected to affect all UV-inducible phages simultaneously. The ability of cells containing the cloned c1 gene to support the induction of heteroimmune prophages argues against a generalized inhibitory effect upon the activation of the RecA protein.

In addition to effects on induction of lambda prophage, pKML11 was capable of producing apparent immunity in E. coli to wild-type and certain clear-plaque mutants of coliphage lambda. The cloned D3 c1 gene may produce immunity to phage lambda by one of several mechanisms. First, this plasmid could contain D3 DNA coding for a phage-specific restriction-modification system (48). Second, the presence of pKML11 could act to reduce the apparent phage titer by increasing the frequency of lysogenization upon initial infection by antagonizing a host-encoded function (66). Third, the presence of the D3 c1 gene product in E. coli AB1157 might inhibit the plating efficiency of coliphage lambda by a direct interaction of the D3 repressor, or at least a heterodimer of lambda cI and D3 c1 proteins, with

lambda DNA. The first two mechanisms would demonstrate non-specific effects on the efficiency of plating of temperate phages while the third would be expected to be specific for lambda.

The third of these hypotheses seems to provide the best explanation for the effects observed. It is supported by the observation that the effects of the D3 c1 gene in E. coli are specific for lambda phages having wild-type promoters. While cI$^+$ and cI857 lambda phages were inhibited by the presence of pKML11, lambda vir and imm^{434} were capable of plating at normal efficiencies. These data suggest that the D3 c1 repressor may be capable of specifically interacting with P_R and P_L of phage lambda to repress the lytic functions of the phage.

The first two mechanisms are considered unlikely because they are not supported by the phage-specific nature of the results obtained. A restriction system similar to that encoded by phage P1 (48) would inhibit the plating of any virus whose genome contained recognition sites for the restriction endonuclease. However, lambda vir and imm^{434} were capable of normal production of virus in the presence of pKML11 and, thus, were not restricted. Likewise, when a clone of the lambda cIII gene is introduced into hflA$^+$ strains of E. coli (21), the increase in lysogen formation due to the

antagonism of the HflA protein function is observed with phages other than lambda, including imm^{434}, which are sensitive to regulation by the HflA protein (66). The cloned D3 c1 gene did not reduce the plating efficiency of imm^{434} phage. In addition, one would not expect a clone of a cII- or cIII-like gene to produce the observed suppression of the clear-plaque phenotype of lambda cI857 at the nonpermissive temperature.

It is clear that the recA gene has been disseminated widely and conserved throughout the eubacteria (11,40,78,101,125). It has been suggested that lambda and certain other phages have evolved to capitalize upon the potential of the RecA protein to monitor the level of DNA damage to the host cell (111). It seems unlikely that the evolution of phages lambda and D3 has been convergent since the closely related phages lambda, 434, and P22 which are all regulated by RecA do not demonstrate cross-immunity. However, phage lambda of E. coli and D3 of P. aeruginosa may have evolved from a common ancestor in a manner similar to that proposed by Campbell and Botstein (17). The phages may have retained functional similarities in their repressor proteins due to the advantages of being able to respond to RecA surveillance of the host cell DNA. Thus one may

hypothesize that while the phages have long since diverged in many ways, radical change in repressor structure and function has been suppressed in order to retain responsiveness to the RecA protein.

CHAPTER VI

INVESTIGATION OF THE lesB908 MUTATION

The establishment of lysogeny upon infection of P. aeruginosa by temperate bacteriophages is affected by at least two genetically distinct host functions. Miller and Ku (96) isolated several P. aeruginosa mutants that were impaired in their ability to be lysogenized. Mutations affecting lysogeny establishment map to at least two distinct loci in the P. aeruginosa chromosome, lesA at approximately 25 min and lesB at approximately 40 min. These mutations do not result in precisely identical phenotypes. A subset of the Les$^-$ mutations (e.g., lesB908) are also recombinationally deficient. The remainder support homologous recombination at essentially wild-type levels. Cells containing the lesB908 mutation are more sensitive to UV and X-ray irradiation. These cells are also impaired in host cell reactivation of infecting damaged bacteriophage (96). These observations

suggest that the effects of the lesB908 mutation may be due to mutation of the P. aeruginosa recA gene. While no data is available concerning the mechanism of interaction of host and phage functions in the formation of lysogens of P. aeruginosa, it is clear from the data of Miller and Ku (96) that the host does have some role in the process. The Les$^-$ phenotype of the cells may be overcome by infecting with phage at high moi suggesting that a phage-encoded gene product required for the establishment of lysogeny is not produced, produced inefficiently, degraded, or inactivated in lesB908 cells. In order to investigate the hypothesis that lesB908 is an allele of the recA gene, a recombinant plasmid carrying the P. aeruginosa recA analogue was mobilized into the lesB908-containing strain of P. aeruginosa, RM8, and the resultant clones examined for complementation of the mutant phenotypes. In addition, a clone of the D3 repressor, c1, was introduced into RM8 to test if overproduction of this repressor would suppress the Les$^-$ phenotype.

Materials and Methods

Bacteria, plasmids and bacteriophage.

The bacterial strains used are listed in Table 21.

Table 21. Bacterial strains.

Strain	Plasmid	Relevant genotype[a]												Prophage[a]	Source or reference
		rec	arg	his	hsd	ilv	leu	lys	pro	pur	sup	trp	thi		
PAO25		+	F10	+	+	+	-10	+	+	+	+	+	+		(43)
PAO303		+	B21	+	+	+	+	+	+	+	+	+	+		(96)
JC9005	FP2	+	+	+	+	+	+	+	+	-600	+	+	+		(96)
RM8		(lesB908)	B21	+	+	+	+	+	+	+	+	+	+		(96)
RM265		-102	+	+	+	+	-10	+	+	+	+	+	+		PAO25
RM2137[b]	pKML6	(lesB908)	B21	+	+	+	+	+	+	+	+	+	+		RM8
RM2138[b]	pKML6	(lesB908)	B21	+	+	+	+	+	+	+	+	+	+		RM8
RM4114	pKML3001	(lesB908)	B21	+	+	+	+	+	+	+	+	+	+		RM8

(Table 21: Continued)

Strain	Plasmid	Relevant genotype[a]												Prophage[a]	Source or reference
		rec	arg	his	had	ilv	leu	lys	pro	pur	sup	trp	thi		
RM5003		+	B21	+	+	+	+	+	+	+	+	+	+	(D3)	PAO303
RM5004	(lesB908)		B21	+	+	+	+	+	+	+	+	+	+	(D3)	RM8
RM5005	pKML3001(lesB908)		B21	+	+	+	+	+	+	+	+	+	+	(D3)	RM8

[a] Genotype symbols and abbreviations are as specified by Bachmann (3) except fon which indicates resistance to phage F116L. Prophage are symbolized by including the name of the phage in parenthesis when present.

[b] RM2137 and RM2138 are two independently isolated transformants of RM8.

Bacteriophages D3 and F116L are temperate phages which respond to the Les$^-$ phenotype (96). Plasmid pKML3001 contains the *P. aeruginosa recA* gene in a 2.3 kbp fragment of *P. aeruginosa* chromosomal DNA cloned into pCP13 (see Chapter IV). Plasmid pKML6 contains the *P. aeruginosa* phage D3 *c1* repressor gene cloned into pME292 (see Chapter V). FP2 is an Inc P-8 fertility factor which mobilizes the *P. aeruginosa* chromosome clockwise from 0 min on the genetic map (96).

Efficiency of lysogenization testing.

(a) Qualitative tests.

These tests were carried out as described by Miller and Ku (96). A small aliquot of phage lysate (approximately 10^9 PFU/ml) was streaked on an L-agar plate and allowed to dry. Cells to be tested were grown in LB and streaked perpendicularily to the phage and the plate incubated at 37^{o}C for 16 h. The Les$^-$ phenotype is demonstrated by the lack of growth at the phage-bacteria streak interface.

(b) Quantitative Les phenotype testing.

Cells to be tested were grown in LB at 37^{o}C to approximately 20 $Klett_{660}$ units. The cells were harvested by centrifugation and suspended in an equal volume of TMN buffer [15 mM NaCl, 10 mM $MgSO_4$ $7H_2O$, 10 mM Tris HCl (pH 7.4)]. The cells were mixed with phage at

several different moi's and incubated at 37°C for 10 min. The infected cells were harvested by centrifugation in an Eppendorf Microfuge and suspended in an equal volume of TMN buffer. The cells were diluted in TMN buffer and plated on L-agar to determine survival. The titer of the phage stock was checked on P. aeruginosa PAO303 to determine the exact moi. Colonies appearing after overnight incubation at 37°C were patched onto fresh L-agar and after a short period of growth, replica-plated onto L-agar seeded with approximately 10^7 CFU of P. aeruginosa PAO303 in 2 ml of lambda top agar. Lysogenized clones were identified by the spontaneous release of phage which caused a clearing in the phage-sensitive PAO303 lawn.

Other methods.

Triparental matings, transformations, conjugations, preparation of phage stocks, induction of prophage and determination of UV sensitivity were done as described in the previous chapters.

Results

Introduction of pKML3001 and pKML6 into RM8.

Plasmid pKML3001 which contains the P. aeruginosa

recA analogue, was introduced into RM8 as described in Chapter IV. Tetracycline resistant transconjugants were tested for UV irradiation sensitivity and for the ability to grow in medium containing 0.01% (v/v) MMS. Plasmid pKML6 carries the phage D3 repressor gene c1 and was introduced into RM8 by transformation as described in Chapter V. Carbenicillin resistant transformants were characterized further. Both plasmids were then tested for the ability to complement the pleiotropic effects of the lesB908 mutation.

UV resistance.

The ability of the plasmids pKML3001 and pKML6 to restore resistance to UV irradiation to P. aeruginosa RM8 was tested. The presence of the cloned recA analogue restored resistance to levels exceeding those exhibited by PAO303, the isogenic $RecA^+$ parent (Figure 26). The presence of plasmid pKML6 did not confer a UV-resistant phenotype to cells containing it (Figure 27).

Conjugational proficiency.

The recombinational ability of lesB908 mutants containing pKML3001 and pKML6 was tested after mating with the FP2 donor strain JC9005 (Table 22). The cloned recA gene almost completely restored recombinational proficiency to RM8 cells containing it. The presence of

Figure 26. Sensitivity to UV irradiation of lesB908 mutants containing pKML3001 clone. Cells were grown to a density of approximately 10^8 CFU/ml in Luria Broth, pelleted, suspended in saline, and exposed to various fluences of UV irradiation. After appropriate dilution in saline, the irradiated cells were plated in duplicate on L-agar and incubated at 37° overnight in the dark. Experiments were performed twice. Mean values are plotted, range is indcated by bars. (●) PAO303 (Rec^+); (■) RM8 (lesB908); (□) RM4114 (lesB908, pKML3001).

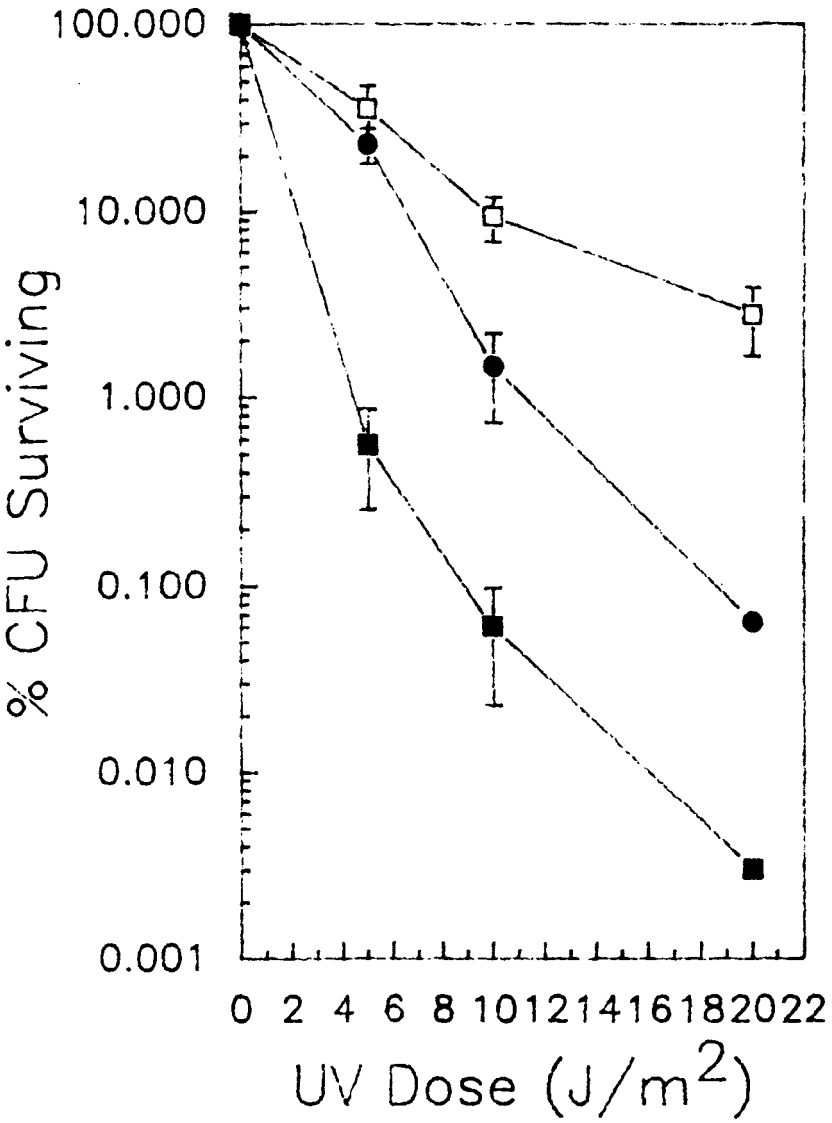

100.000
10.000
1.000
0.100
0.010
0.001
% CFU Surviving
0 2 4 6 8 10 12 14 16 18 20 22
UV Dose (J/m2)

Figure 27. Sensitivity to UV irradiation of lesB908 mutants containing the D3 c1 clone. Cells were grown to a density of approximately 10^8 CFU/ml in Luria Broth, pelleted, suspended in saline, and exposed to various fluences of UV irradiation. After appropriate dilution in saline, the irradiated cells were plated in duplicate on L-agar and incubated at 37° overnight in the dark. Experiments were performed twice. Mean values are plotted, range of data is indicated by bars. (●) PAO303 (Les^+); (■) RM8; (lesB908) (□) RM2137 (lesB908, pKML6).

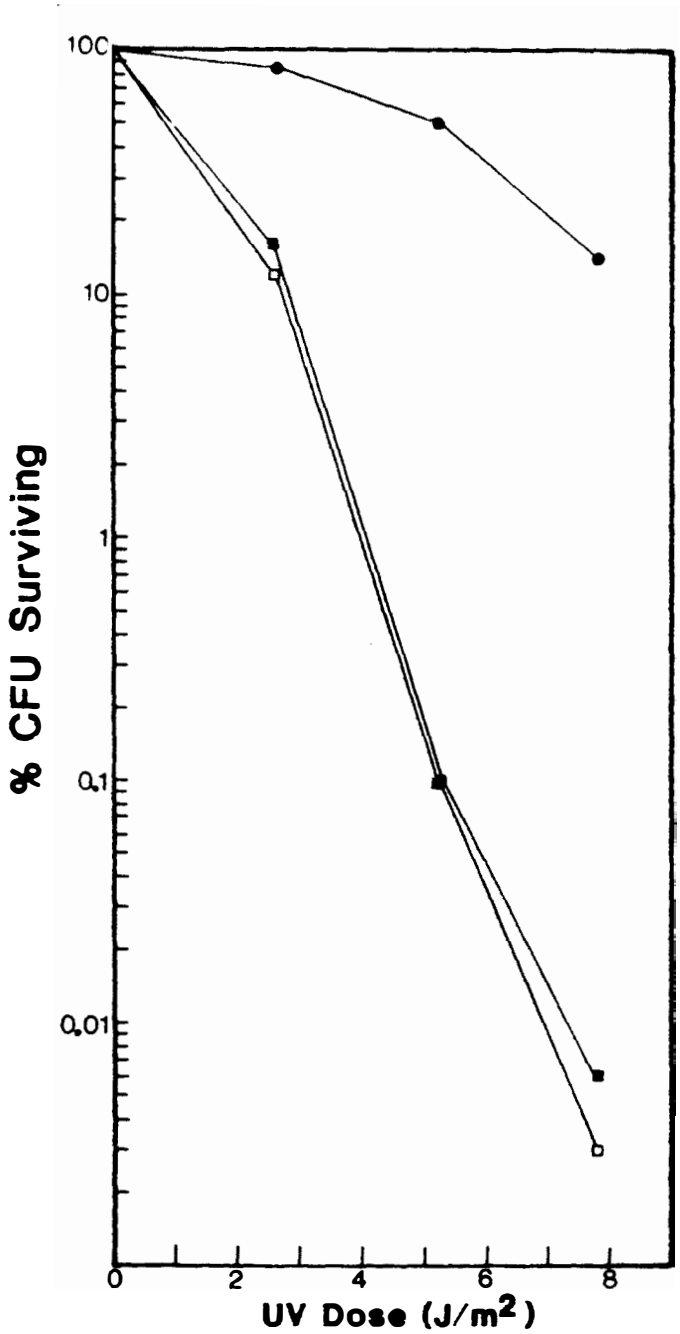

100
10
1
0.1
0.01
% CFU Surviving
0
2
4
6
8
UV Dose (J/m2)

Table 22. Recombinational proficiency of *lesB908* mutants containing pKML3001 and pKML6[a].

Strain	Relevant Characteristics		Recombinational Proficiency (*argB*$^+$ recombinants / 10^6 donors)
PAO303	Rec$^+$	(1)[b]	5.80
		(2)	8.75
RM8	*lesB908*	(1)	<0.02
		(2)	<0.02
RM4114	*lesB908*; pKML3001[c]	(1)	1.50
		(2)	2.30
RM2138	*lesB908*; pKML6	(1)	<0.02
		(2)	<0.02

[a]JC9005 was used as the donor strain. Cells were mixed in a donor-to-recipient ratio of 1:1.2. Matings were performed for 2 h at 37°C without shaking.

[b]Experiments were done two times. Platings were done in duplicate.

[c]pKML3001 contains the *P. aeruginosa recA* analogue. pKML6 contains the D3 *c1* gene.

plasmid pKML6 did not allow P. aeruginosa RM8 to support homologous recombination.

Prophage induction.

The levels of spontaneous and UV-stimulated induction to lytic growth of D3 prophage in lysogens of RM8 were determined. The presence of plasmid pKML3001 increased the level of spontaneous phage D3 release from lysogens to the level found after UV induction of wild-type P. aeruginosa cells (Table 23). However, no increase in the amount of phage released subsequent to exposure of the cells containing pKML3001 to UV irradiation was apparent.

Efficiency of lysogenization.

Qualitative Les tests performed on P. aeruginosa RM8 with and with out pKML3001 revealed an apparent suppression of the Les$^-$ phenotype by plasmid pKML3001 (Figures 28 and 29). The frequency of the establishment of lysogeny in RM8 with and without pKML3001 after infection by phage F116L was quantitated (Figure 30). The presence of the cloned recA gene restored the ability of the cells to be lysogenized, although the frequency did not reach wild-type levels at equivalent MOI's.

The D3 c1 gene clone was introduced into RM8 to determine if the Les$^-$ phenotype could be suppressed by increasing the gene-dosage of the phage repressor. The

Table 23. Induction of D3 prophage[a]

Strain	Relevant Characteristics		Viability (CFU/ml)	Phage titer (PFU/10^4 CFU) Spontaneous	Induced
RM5004	lesB908	(1)[b]	4×10^7	1	1.5
		(2)	4×10^7	2.5	2.5
RM5005	lesB908; pKML3001[c]	(1)	7×10^7	23,000	33,000
		(2)	7×10^7	29,000	29,000
RM5003	Rec^+	(1)	7×10^7	240	14,000
		(2)	7×10^7	290	110,000

[a]Lysogens were induced by exposing to 10 J/m^2 and incubating for 2 h at 37^o as described in Chapter IV.

[b]Experiments were done two times. All platings were done in duplicate.

[c]pKML3001 contains the P. aeruginosa recA analogue.

Figure 28. Qualitative Les test with phage F116L. Strains were cross-streaked against phage F116L as described in the text. (A) RM8 (lesB908), (B) PAO303 (les^+), (C) RM4114 (lesB908, pKML3001) (D) RM265 (rec-102).

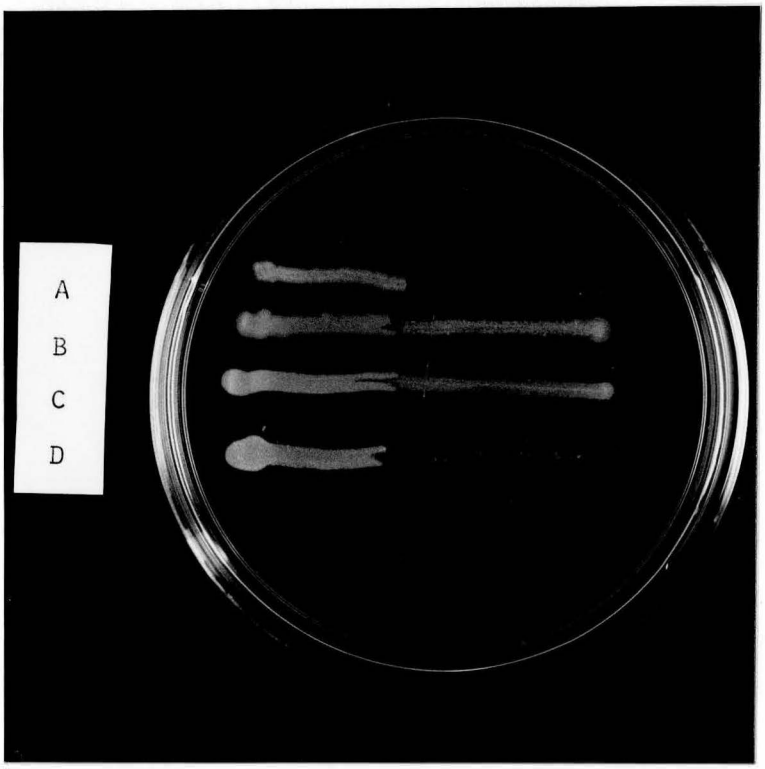
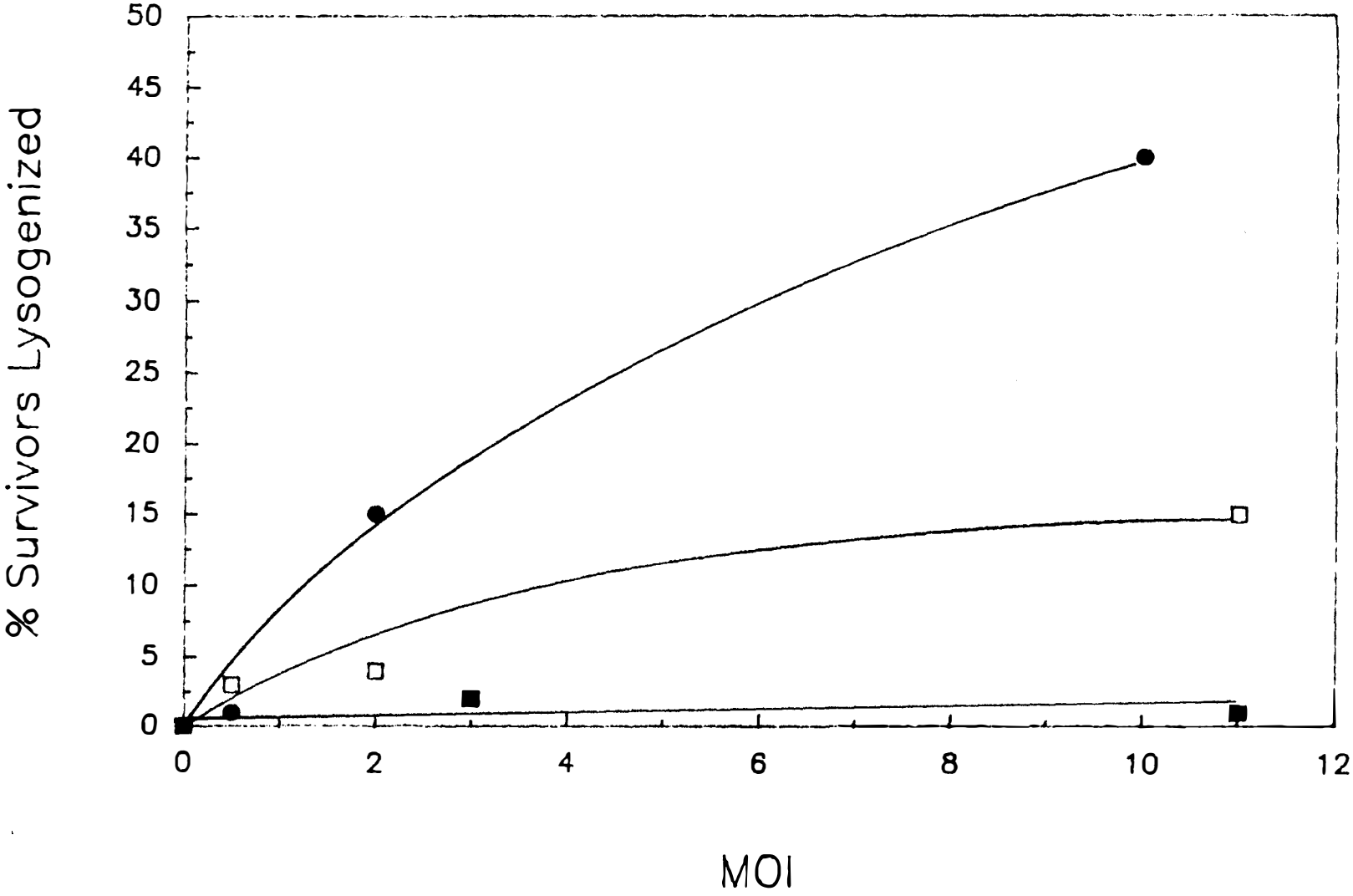

A
B
C
D

Figure 29. Qualitative Les test with phage D3. Strains were cross-streaked against phage D3 as described in the text. (A) RM8 (lesB908), (B) PAO303 (Les^+), (C) RM4114 (lesB908, pKML3001), (D) RM265 (rec-102).


A
B
C
D

Figure 30. Efficiency of lysogenization of lesB908 mutants containing pKML3001. Strains were infected with temperate phage F116L at various MOI's and the percentage of total input cells lysogenized was measured as described in the text. A representative experiment of two repetitions is shown. The data obtained in both sets of experiments are qualitatively the same. (●) PAO303 (Les^+); (■) RM8 (lesB908); (□) RM4114 (lesB908, pKML3001).

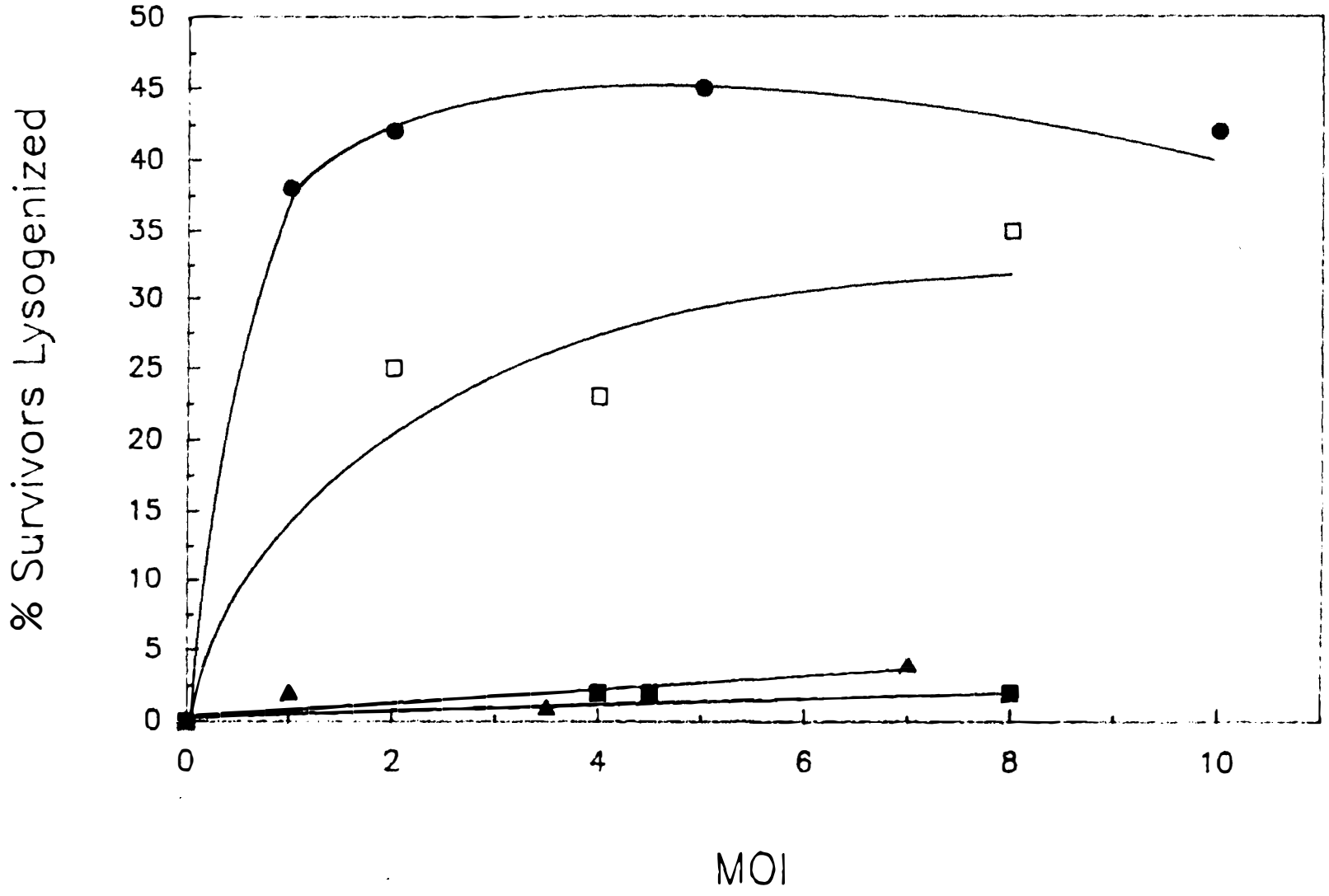

% Survivors Lysogenized
50
45
40
35
30
25
20
15
10
5
0
0
2
4
6
8
10
12
MOI

presence of plasmid pKML6 in two independently transformed clones conferred a Les^+ phenotype to RM8 cells containing it (Figure 31). This suppression of the Les^- phenotype is specific for cells infected by phage D3 as the frequency of lysogenization by F116L was not increased (Figure 31).

Discussion

The recA-complementing plasmid pKML3001 restores recombinational proficiency and resistance to UV irradiation to P. aeruginosa RM8, supporting the hypothesis that lesB908 is an allele of the P. aeruginosa recA gene. The phenotypic effects of the lesB908 mutation may be best explained by assuming that the lesB908 gene product is altered in such a way that it has lost synaptase activity but possesses protease activity intrinsically even in the absence of DNA damage. It is further hypothesized that the mutant protein has reduced affinity for single-stranded DNA and that, through a cooperative interaction between wild-type and mutant proteins, the affinity of LesB908 protein for single-stranded DNA is enhanced when wild-type RecA protein is also present in the cell.

Figure 31. Efficiency of lysogenization of lesB908 mutants containing pKML6. Strains were infected with temperate phage D3 or F116L at various MOI's and the percentage of total input cells lysogenized was measured as described in the text. A representative experiment of two repetitions is shown. The data obtained in both sets of experiments are qualitatively the same. (●) PAO303 (Les$^+$) [phage D3]; (■) RM8 (lesB908) [phage D3]; (□) RM2137 (lesB908, pKML6) [phage D3]; (▲) RM2137 (lesB908, pKML6) [phage F116L].


% Survivors Lysogenized
50
45
40
35
30
25
20
15
10
5
0
0
2
4
6
8
10
MOI

In E. coli, alleles of the recA gene which encode recombination-deficient, protease-constitutive mutant proteins have been reported (132). Strains containing these recA mutations phenotypically resemble P. aeruginosa lesB908 mutants in that they are sensitive to UV irradiation and inactivate certain repressor proteins in the absence of DNA damage. Unfortunately, experiments examining the effects of addition of a wild-type recA gene to these cells have not been performed. An examination of the effects of these mutations upon lysogeny establishment has not been reported.

Another allele of the E. coli recA gene (recA142) which also resembles the lesB908 mutation of P. aeruginosa has been well characterized. Cells containing the recA142 allele are recombination-deficient and, if lysogenic, spontaneously release phage in contradistinction to strains containing other recA mutations (25,111). In addition, recA142 strains are incapable of inducing higher levels of phage release after exposure of the lysogens to DNA damaging agents. The effects of recA142 upon lysogeny establishmnet have not been reported.

The RecA142 protein has been well characterized in vitro and may provide some insights into the

understanding of how the lesB908 mutation causes its effects. In vitro RecA142 protein exhibits a low level of spontaneous protease activity toward lambda cI repressor but binds single-stranded DNA very poorly (S. Kowalczykowski, personal communication). This is intriguing since single-stranded DNA is presumed to be the signal required for activation of RecA protein to the proteolytic state (111). In the absence of wild-type RecA protein, RecA142 protein binds single-stranded DNA only inefficiently and the full potential for protease activity is not observed. Addition of wild-type RecA protein to an in vitro cI-cleavage reaction containing RecA142 protein causes an increase in the rate and extent of cI cleavage as well as an increase in the RecA copolymer's ability to bind single-stranded DNA (S. Kowalczykowski, personal communication). Such cooperativity and codominance of alleles is a hallmark of the RecA protein. It has been hypothesized that wild-type RecA protein is capable of binding to single-stranded DNA and cooperatively promotes the binding of RecA142 protein. The more efficient binding of the RecA142 protein to single-stranded DNA increases the expression of its protease activity.

The introduction of plasmid pKML3001 into P. aeruginosa RM8 (lesB908) has several very interesting

effects which are reminiscent of the *in vitro* observations made using mixtures of wild-type and RecA142 protein. The introduction of the *P. aeruginosa* wild-type *recA* allele on plasmid pKML3001 into RM8 complements UV resistance to greater than wild-type levels (Figure 25). This may be a cooperative effect due to the presence of the gene products of both the wild-type *recA* and *lesB908* alleles. If the expression of *recA* is inducible and autoregulated in *P. aeruginosa* in a manner analogous to that of the *E. coli recA* gene, the introduction of a regulated wild-type allele on plasmid pKML3001 into a *lesB908*-containing mutant would be expected to lead to overexpression of the wild-type protein. It is clear that in *E. coli* the RecA protein effects the level of cell survival subsequent to UV irradiation both by regulating gene expression of the SOS network (including the *recA* gene itself) and by acting in some uncharacterized capacity in several DNA repair systems (109,132). Overproduction of wild-type RecA protein could lead to hyper-UV resistance through increased activity of RecA-dependent repair pathways. The data presented in Chapter III for fusion plasmids in $RecA^+$ *E. coli* strains as well as the identification of an SOS box consensus sequence in the 5' leader sequence of the *P.*

aeruginosa recA gene by M. Kageyama (personal communication) suggest induction of the gene may occur. However, the fusion plasmid data is subject to other interpretations and nothing is known of the existence of a lexA analogue in P. aeruginosa so this explanation must be regarded as strictly hypothetical. Also it is possible that the recA gene is merely expressed at a moderate, constitutive, level and is not regulated at all in P. aeruginosa. In order to determine whether or not this is the case, it would be necessary to examine the UV resistance of wild-type P. aeruginosa containing pKML3001 to determine if the cells become UV super-resistant.

The hypothesis that P. aeruginosa does contain some form of a DNA-damage inducible (i.e. SOS) network is consistent with the observations of Benbrook and Miller (10) who demonstrated that while neither wild-type nor lesB908 P. aeruginosa contained a quinolone-inducible error-prone DNA repair system, stable DNA synthesis (an SOS function in E. coli) was inducible in RecA$^+$ P. aeruginosa but was constitutively expressed in RM8. In addition, Horne and Ohman (J. M. Horne and D. E. Ohman, Abstr Annu. Meet. Am. Soc. Microbiol. 1987, H130, p.161) showed that a P. aeruginosa recA-chloramphenicol acyl transferase fusion was inducible by MMS in P. aeruginosa. Therefore, the overexpression of a wild-type allele of

the recA gene in lesB908 cells could be significant for cell survival after exposure to DNA-damaging agents even if P. aeruginosa lacks an inducible error-prone DNA repair system.

lesB908-containing mutants support spontaneous induction of prophage D3 at reduced but significant levels (Table 22). This is again reminiscent of the recA142 allele of the E. coli recA gene. It is clear that in both E. coli and P. aeruginosa DNA damage-activated recA gene products interact with phage repressors and promote their inactivation. This interaction must also be ongoing in the absence of inducing treatments since the spontaneous release of phage also requires functional recA gene product, at least for most recA alleles. Such spontaneous induction of prophage may indicate that the intrinsic level of DNA damage in a subpopulation of cells is high enough to induce activation of RecA protein in those cells. In the absence of wild-type RecA the proteolytic activity of lesB908-containing cells is lowered due to the weak binding of homopolymers of the mutant protein to single-stranded regions of DNA. Upon introduction of plasmid pKML3001, the level of spontaneous prophage induction in RM8 is significantly increased to levels equal to the

fully UV-induced state of the wild-type isogenic parental strain. This, too, suggests a codominant cooperative effect between the wild-type RecA and LesB908 proteins which potentiates the effective proteolytic activity of the mutant protein. A cooperative interaction between wild-type and mutant RecA proteins may allow an enhanced level of binding to single-stranded regions of DNA formed by spontaneous damage to the host's DNA. The increased sensitivity of surveillance of DNA damage by the $RecA^{+}$-LesB908 heteropolymer may potentiate protease activity to levels promoting prophage induction in an increased fraction of the lysogenic population. This in turn increases the spontaneous levels of phage production in an actively growing culture even to levels above those observed in the wild-type strain.

When a functional allele of the P. aeruginosa recA analogue is introduced into a lesB908 mutant, the Les^{-} phenotype is partially complemented allowing establishment of lysogeny by phage F116L at increased levels. Hence, it appears that the Les^{-} phenotype is a pleiotropic consequence of certain alleles of the P. aeruginosa recA gene. The exact nature of the mechanism leading to the Les^{-} phenotype in lesB908-containing mutants is unknown.

In the wild-type cell, after infection a decision

is made by the phage to grow lytically or temperately. This decision is influenced in part by the amount of phage-specific repressor initially synthesized in the cell. Presumably, a number of host and phage functions interact to determine this level of repressor. It is clear from the data presented here that one level of interaction between the P. aeruginosa RecA protein and prophages is as an inducing agent after UV irradiation (see Chapters IV and V) and that the mechanism of prophage induction is probably identical for lambda of E. coli and D3 of P. aeruginosa (Chapters II and III).

The lesB908 mutation appears to lead to production of a modified RecA protein which constitutively expresses a low-level repressor-inactivating activity. Temperate phages infecting such a cell would enter the lytic pathway due to the heightened instability of newly synthesized repressor protein. The Les$^-$ phenotype may be overcome by elevating the total amount of phage repressor protein synthesized (i.e. by increasing the copy-number of the repressor gene by addition of pKML6 to the cell or by increasing the MOI) which suggests that the initial rate of repressor accumulation may be the crucial determinant of the pathway of development selected by the phage. Because of its constitutively activated state,

the LesB908 protein acts to destroy repressor protein upon its synthesis increasing the probability of lytic growth and cell lysis.

The level of complementation of the Les$^-$ phenotype by the introduction of pKML3001 into RM8 is not to fully wild-type levels again suggesting the codominance of the wild-type and lesB908 alleles. In the establishment of lysogeny the synthesis of repressor (a phage gene product) begins upon infection and must reach a threshold level in a finite time period in order for lysogenization of the infected cell to take place. The protease-constitutive lesB908 protein may reduce the rate of active repressor accumulation through inactivation of the newly synthesized phage protein and thereby reduce the probability of threshold repressor concentrations being reached in the requisite time period. The presence of plasmid pKML3001 may allow the complementation of the Les$^-$ phenotype by producing wild-type recA gene product (not activated) which competes with the mutant (activated) protein for phage repressor. Repressor molecules interacting with wild-type, non-activated, RecA molecules may be protected from destruction by the LesB908 protein. Hence, the effective rate of accumulation of repressor may be increased allowing threshold levels to be achieved in a statically larger

subpopulation of infected cells. This effect would allow partial complementation of the Les$^-$ phenotype. This hypothesis is consistent with the observations that increasing the number of copies of the phage repressor gene by increasing the MOI (i.e. average number of phage infecting a given cell) or introducing the cloned phage repressor gene will also supresses the Les$^-$ phenotype of RM8 in a phage-specific fashion. It is certainly clear from these observations as well as those made in Chapter V that minor perturbations in the net rate of repressor accumulation may have dramatic effects upon the ability of an infecting phage to carry out the lysis-lysogeny decision-making process and on the outcome of this process.

The exact nature of the mutation leading to the Les$^-$ phenotype associated with the lesB908 mutation remains unclear. A total understanding of the mechanism of this phenomenon and the role or roles played by the P. aeruginosa recA protein must await the purification and in vitro characterization of this protein from RecA$^+$ and lesB908 strains of P. aeruginosa.

CHAPTER VII

CONCLUSIONS

The recA gene, whatever its origin, appears to have been conserved in the eubacteria (11,40,49,78,94,101,125). Analogues have been found in several genera some of which have been shown to have DNA base sequence homology to the E. coli recA gene (11,78,125). The data presented in this dissertation demonstrate that P. aeruginosa contains a recA gene which is homologous structurally and functionally to the recA gene of E. coli. Homology at the DNA sequence level was revealed by Southern analysis at high stringency. In addition, Western blotting experiments have demonstrated the P. aeruginosa gene product cross reacts with anti-E. coli recA antibody and shares homology at the amino acid sequence level to a region or regions of the E. coli protein (S. Kowalczykowski, personal communication). The E. coli recA-complementing activity was found to be

contained within a 2.3 kb BamHI-HindIII fragment of DNA. Ultimately, the complementing activity was found to be contained within a 1.5 kb HindIII-PvuII DNA fragment.

Experiments revealed that P. aeruginosa strains containing rec-102 and lesB908 were complemented to recombinational proficiency by plasmids containing the BamHI-HindIII DNA fragment. The recA gene carries out similar functions in both E. coli and P. aeruginosa. Both bacterial species require RecA to perform homologous recombination.

Induction of certain prophages subsequent to DNA damaging treatments requires the presence of a functional recA gene product (111). In E. coli the induction to lytic growth of prophage lambda is the result of the specific cleavage of the phage cI repressor at an Ala-Gly peptide bond (112). The P. aeruginosa recA gene product supports the induction of prophage lambda in E. coli recA mutants by a similar mechanism (Chapter III). The induction of the P. aeruginosa phage D3 was shown to be recA-dependent. The recombination-deficient mutations rec-102 and lesB908 inhibited this UV induction of prophage D3. The defect in mutants containing rec-102 and lesB908 was complemented by the recA-containing plasmid.

In addition to its role in homologous recombination, RecA also has a role in the repair of DNA damage in P. aeruginosa. The loss of recA function in mutants containing rec-102 and lesB908 clearly sensitizes the cell to both ionizing and nonionizing (96) radiation as well as to certain chemicals which damage DNA. The P. aeruginosa recA clone complements the UV damage repair defects associated with recA mutations in both P. aeruginosa and E. coli.

Also noteworthy is the conservation of the pattern of expression of the recA gene between P. aeruginosa and E. coli. The fact that the P. aeruginosa recA gene is expressed at all in E. coli argues for some sort of conservation of promoter structure in the gene. Normally, P. aeruginosa genes are only poorly expressed in E. coli (73). However, the data presented indicate that the P. aeruginosa recA promoter functions well in E. coli. Whether the expression of the P. aeruginosa gene is under the control of the LexA protein in E. coli in the same fashion as the E. coli recA gene is unclear. The apparent control of expression by E. coli LexA protein has been shown for the recA analogues of certain enteric bacterial strains (125). The DNA sequence of the P. aeruginosa recA gene has revealed the presence of an SOS box consensus sequence (M. Kageyama, personal

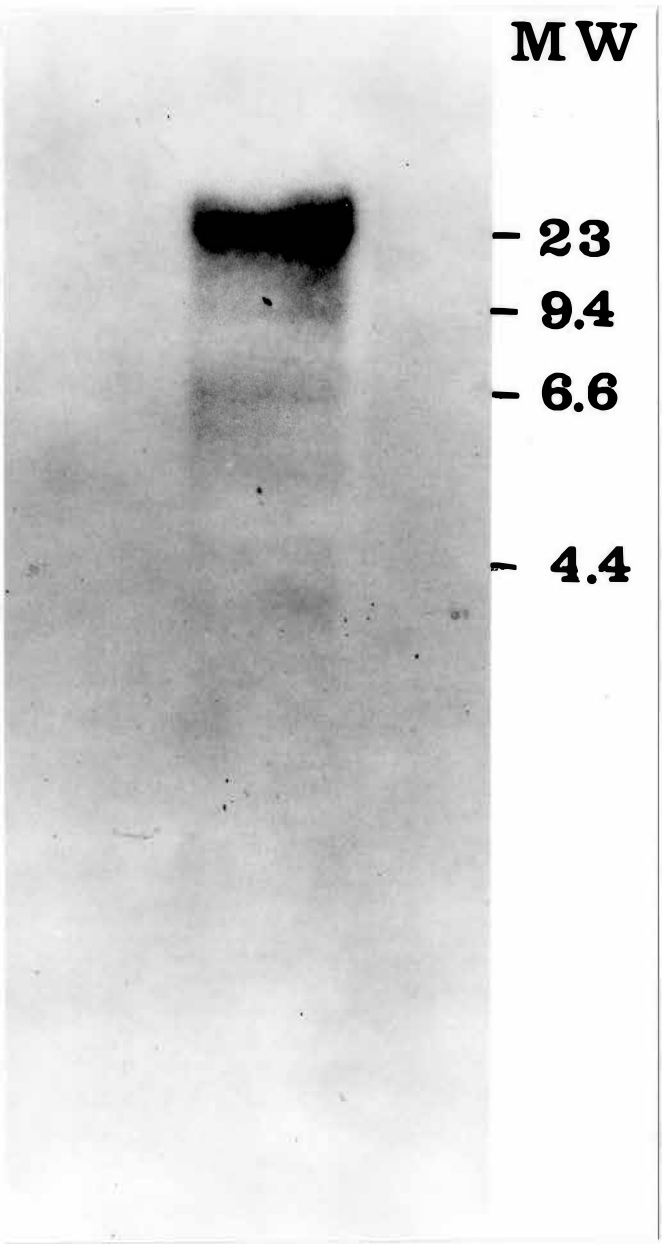
communication). It is unknown whether a homologous analogue to the lexA gene exists in P. aeruginosa, but these considerations argue that there could possibly be such a gene expressed in P. aeruginosa.

While the two genes and their protein products are similar, they are not precisely identical. The chromosomal DNA fragments encoding the two genes have different restriction endonuclease maps. The two protein products also seem to be dissimilar. The P. aeruginosa gene product is larger than the E. coli protein. This size differential was revealed by SDS polyacrylamide gel electrophoresis of the P. aeruginosa protein expressed in E. coli and comparison to the literature values for the E. coli protein. Also, Western analysis of the P. aeruginosa protein using anti-E. coli RecA antibody has directly confirmed that the P. aeruginosa recA gene product is larger than the E. coli recA gene product (S. Kowalczykowski and R. V. Miller, personal communications).

P. aeruginosa is lysogenized by several phages capable of being induced by UV irradiation in a process requiring RecA. One of these temperate phages, D3, has been examined in more detail. This phage produces a repressor of lytic functions that appears to be a

functional analogue of phage lambda cI protein and is capable of interacting directly with phage lambda phage operators to control the expression of lambda genes. While the two phages are not homologous at the DNA sequence level under conditions of high stringency, the two repressor proteins from these phages clearly perform similar functions. Perhaps the phages did evolve from a common ancestor with the repressor structure conserved to retain reactivity to RecA present in both bacterial species. Campbell and Botstein (17) have proposed that lambda-like phage evolution may occur by a constant reshuffling by recombination of host or defective prophage genes between functional superinfecting viruses and the host chromosome. While it is unexpected that a bacterial species such as P. aeruginosa would contain lambda homologous DNA sequences, plasmid pKML2003 does exhibit DNA sequence homology to phage lambda under conditions of high stringency (Figure 32) indicating the possibility that in evolutionary history, P. aeruginosa has been host to a lambda-like phage. It is unclear just what portion of pKML2003 is homologous to phage lambda. No experiment can rule out the possibility that this DNA sequence homology is due to convergent evolution, but the presence of lambda-like DNA in P. aeruginosa is intriguing.

Figure 32. Southern analysis of homology between P. aeruginosa chromsomal and lambda phage DNAs. This blot was probed with nick translated pKML2003 DNA. Hybridization to a HindIII digest of phage lambda DNA was carried out under conditions of high stringency.


MW
23
9.4
6.6
4.4

The alteration of the P. aeruginosa recA gene responsible for producing the Les⁻ phenotype associated with lesB908 appears to produce a RecA protein which is a more active inducing agent. The data presented here and in the work of Benbrook and Miller (10) suggest that the proteolysis-stimulating activity of the P. aeruginosa RecA protein is constitutively expressed at a low level in lesB908 mutants. Similar mutations of the E. coli recA gene have recently been identified by Tessman and Peterson (132). While the exact mechanism of the RecA-mediated induction of phage in P. aeruginosa has yet to be determined, the data presented in this dissertation suggest that the mechanism is quite similar to the induction of lambda prophage by activated E. coli RecA protein. Certainly the induction-promoting function of P. aeruginosa RecA protein is involved in producing the Les⁻ phenotype in RM8.

The potential of P. aeruginosa to repair damage to DNA is presently under investigation. The data presented in this dissertation reiterate that P. aeruginosa is much more sensitive to UV irradiation than E. coli. It is possible that P. aeruginosa lacks certain functions having a role in DNA repair that are present in E. coli. Experimentation has revealed that P. aeruginosa does not have a DNA damage-inducible mutagenesis system (10, R. V.

Miller, personal communication). The hypothesis may be made that P. aeruginosa lacks certain genetic elements such as umuDC, or these elements, if present, are not expressed. The sensitization of rec-102 and lesB908-containing strains could also be due to the loss of the ability of the P. aeruginosa RecA protein to perform recombinational repair after UV damage rather than the loss of induction of additional DNA repair pathways.

Wild-type strains of P. aeruginosa may possess essentially the same inate ability to resist UV irradiation as E. coli, but this abilty is inapparent due to epistasis. P. aeruginosa is commonly lysogenized by several temperate phages (62). If P. aeruginosa is lysogenized by prophage(s) inducible by UV irradiation, such strains would be apparently more sensitive to exposure to UV. In addition, many strains of P. aeruginosa have been proven to contain within their genetic material UV-inducible pyocins some of which resemble phage tail structures and may represent defective prophage (62,63,121). In particular, pyocin AP41 is interesting because it is inducible by UV irradiation and maps very near the recA locus on the P. aeruginosa PAO chromosome (121). Induction of expression of one or more such genetic elements could lead to the

death of the cell and produce an apparent increase in overall sensitivity of the strain to the inducing agent.

While it is conceivable that P. aeruginosa has a less sophisticated DNA repair network, the data presented in this dissertation raise the possibilty that the DNA repair systems of P. aeruginosa have the potential to be quite similar to those of E. coli. Both P. aeruginosa and E. coli contain recA genes that are clearly homologous to each other. The gene products carry out the same functions in their respective hosts and are vital for cell survival after exposure to DNA damaging treatments.

The mechanism causing the activation of the recA gene product to its proteolytic-promoting state has not been elucidated. However, it is clear that E. coli is capable of generating an activating signal upon exposure to an inducing signal sufficient to cause the activation of the P. aeruginosa recA gene product. Whatever the means of generating the signal, either the mechanism of its creation is conserved or it is an aspect common to both E. coli and P. aeruginosa cells exposed to inducing treatments. Determination of the biochemical nature of this signal in P. aeruginosa and whether or not the recA

gene product controls the expression of other genes in this species should provide avenues for productive future experimentation.

LITERATURE CITED

1. Anderson, E. J. 1949. Growth requirements of virus-resistant mutants of Escherichia coli strain "B". Proc. Natl. Acad. Sci. U. S. A. 32:120-128.

2. Arber, W., L. Enquist, B. Hohn, N. E. Murray and K. Murray. 1983. Experimental methods for use with lambda, p.433 - 466. In R. W. Henrix, J. W. Roberts, F. W. Stahl and R. A. Weisberg (eds.), Lambda II. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

3. Bachmann, B. J. 1983. Linkage map of Escherichia coli K-12, edition 7. Microbiol. Rev. 47:180-230.

4. Bagdasarian, M., A. Bailone, M. M. Bagdasarian, P. A. Manning, R. Lurz, K. N. Timmis, and R. Devoret. 1986. An inhibitor of SOS induction specified by a plasmid locus in Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. 83:5723-5726.

5. Bagdasarian, M., R. D'Ari, W. Filiopwicz and J. George. 1980. Suppression of induction of SOS functions in an Escherichia coli tif-1 mutant by plasmid R100.1. J. Bacteriol. 141:464-469.

6. Bailone, A., A. Levine and R. Devoret. 1979. Inactivation of prophage lambda repressor in vivo. J. Mol. Biol. 131:553-572.

7. Bartlett, D. H. 1985. Ph. D. Dissertation, The University of Illinois at Chicago, Chicago, Illinois.

8. Bartlett, D. H. and P. Matsumura. 1984. Identification of Escherichia coli region III flagellar gene products and description of two new flagellar genes. J. Bacteriol. 160:577-585.

9. Belfort, M. and D. L. Wulff. 1973. Genetic and biochemical investigation of the Escherichia coli mutant hfl-1 which is lysogenized at frequency by bacteriophage lambda. J. Bacteriol. 115:299-306.

10. Benbrook, D. M. and R. V. Miller. 1986. Effects of norfloxacin on DNA metabolism in Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 29:1-6.

11. Better, M. and D. R. Helinski. 1983. Isolation and characterization of the recA gene of Rhizobium melliloti. J. Bacteriol. 155:311-316.

12. Bialkowska-Hobrzanska, H., C. A. Gilchrist and D. T. Denhardt. 1985. Escherichia coli rep gene: Identification of the promoter and N terminus of the Rep protein. J. Bacteriol. 164:1004-1010.

13. Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker and H. W. Boyer. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95-113.

14. Boyer, H. W. and D. Roullard-Dussoix. 1969. A complementation analysis of the restriction and modification of DNA in Escherichia coli. J. Mol. Biol. 41:459-472.

15. Bridges, B. A., and S. G. Sedgwick. 1974. Effect of photoreactivation on the filling of gaps in deoxyribonucleic acid synthesized after exposure of Escherichia coli to ultraviolet light. J. Bacteriol. 117:1077-1082.

16. Brookes, K., and A. J. Clark. 1966. Behavior of lambda bacteriophage in a recombination-deficient strain of Escherichia coli. J. Virol. 1:283-293.

17. Campbell, A. and D. Botstein. 1983. Evolution of the lambdoid phages, p. 365-380. In R. W. Hendrix, J. W. Roberts, F. W. Stahl and R. A. Weisberg (ed.), Lambda II. Cold Spring Harbor Laboratories, Cold Spring Harbor, N. Y

18. Castellazzi, M., J. George and G. Buttin. 1972. Prophage induction and cell division in Escherichia coli. II. Linked (recA zab) and unlinked (lexA) suppressors of tif--mediated induction and filamentation. Mol. Gen. Genet. 119:153-174.

19. Castellazzi, M., P. Morand, J. George and G. Buttin. 1977. Prophage induction in Escherichia coli. V. Dominance and complementation analysis in partial diploids with pleiotropic mutations (tif, recA, zab and lexB) at the recA locus. Mol. Gen. Genet. 153:297-310.

20. Casthilo, B. A., P. Olfson and M. J. Casadaban. 1984. Plasmid insertion mutagenesis and lac gene fusion with mini-mu bacteriophage transposons. J. Bacteriol. 158:488-495.

21. Cavenagh, M. M. and R. V. Miller. 1986. Specialized transduction of Pseudomonas aeruginosa PAO by bacteriophage D3. J. Bacteriol. 165:448-452.

22. Chamberlain, J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with water soluble fluor sodium salicylate. Analyt. Biochem. 98:132-135.

23. Chandler, P. M., and V. Krishnapillai. 1974. Isolation and properties of recombination-deficient mutants of Pseudomonas aeruginosa. Mutat. Res. 23:15-23.

24. Chow, S. A., S. M. Honigberg, R. J. Bainton and C. M. Radding. 1986. Patterns of nuclease protection during strand exchange. J. Biol. Chem. 261:6961-6971.

25. Clark, A. J. 1973. Recombination deficient mutants of Escherichia coli and other bacteria. Ann. Rev. Genet. 7:67-86.

26. Clark, A. J. and A. D. Margulies. 1965. Isolation and characterization of recombination deficient mutants of Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. 53:451-459.

27. Clark-Curtiss, J. E. and R. Curtiss. 1983. Analysis of recombinanat DNA using Escherichia coli minicells. Methods Enzymol. 101:347-362.

28. Cohen, S., B. Knoll, J. W. Little and D. W. Mount. 1981. Preferential cleavage of phage lambda repressor by recA protease. Nature 294:182-184.

29. Craig, N. L., and J. W. Roberts. 1980. Escherichia coli recA-directed cleavage of lambda repressor requires polynucleotide. Nature 283:26-30.

30. Cunningham, R. P., C. DasGupta, T. Shibata and C. M. Radding. 1980. Homologous pairing in genetic recombination: recA protein makes joint molecules of gapped circular and closed circular DNA. Cell 20:223-235.

31. Darzins, A., and A. M. Chakrabarty. 1984. Cloning of genes controlling alginate biosynthesis from a mucoid cystic fibrosis isolate of Pseudomonas aeruginosa PAO. J. Bacteriol. 159:9-18.

32. DasGupta, C., T. Shibata, R. P. Cunningham and C. M. Radding. The topology of homologous pairing promoted by RecA protein. Cell 22:437-446.

33. Davis, R. W., D. Botstein and J. R. Roth. 1981. A manual for genetic engineering. Advanced bacterial genetics. Cold Spring Harbor Labortory. Cold Spring Harbor, NY.

34. De Anda, J., A. R. Poteete and R. T. Sauer. 1983. P22 c2 repressor. Domain structure and function. J. Biol. Chem. 258:10536-10642.

35. deBruijn, F. J. and J. R. Lupski. 1984. The use of transposon Tn5 in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids - a review. Gene 27:131-149.

36. Defais, M., P. Caillet-Faquet, M. S. Fox and M. Radman. 1976. Induction kinetics of mutagenic DNA repair activity in E. coli following ultraviolet irradiation. Mol. Gen. Genet. 148:125-130.

37. Defais, M., P. Faquet, M. Radman and M. Errera. 1971. Ultraviolet reaction and ultraviolet mutagenesis of lambda in different genetic systems. Virology 43:495-503.

38. Demerec, M., E. A. Adelberg, A. J. Clark and P. E. Hartman. 1966. A proposal for a uniform nomenclature in bacterial genetics. Genetics 54:61-74.

39. Egan, J. B., and B. W. Holloway. 1961. Genetic studies on lysogeny in Pseudomonas aeruginosa. Aust. J. Exp. Biol. 39:9-18.

40. Eitner, G., B. Adler, V. A. Lanzov and J. Hofemeister. 1982. Interspecies recA protein substitution in Escherichia coli and Proteus mirabilis. Mol Gen. Genet. 185:481-486.

41. Emmerson, P. T. and S. C. West. 1977. Identification of protein X of Escherichia coli as the recA$^+$/tif$^+$ gene product. Mol. Gen. Genet. 155:77-85.

42. Friedman, A. M., S. R. Long, S. E. Brown, W. J. Buikema and F. M. Ausubel. Construction of a broad host range cosmid cloning vector and its use in th genetic analysis of Rhizobium mutants. Gene 18:289-296.

43. Früh, R., J. M. Watson and D. Haas. 1983. Construction of reconbination-deficient strains of Pseudomonas aeruginosa. Mol. Gen. Genet. 191:334-337.

44. Ganesan, A. K. 1974. Persistence of pyrimidine dimers during post-replication repair in ultraviolet light-irradiated Escherichia coli K-12. J. Mol. Biol. 87:103-119.

45. Ganesan, A. K., and P. C. Seawell. 1975. The effect of lexA and recF mutations on post-replication repair and DNA synthesis in Escherichia coli K-12. Mol. Gen. Genet. 141:189-205.

46. Gertman, E., G. N. White, D. Berry and A. M. Kropinski. 1986. IS22: a new insertion element associated with the genome of Pseudomonas aeruginosa. J. Bacteriol. 166: 1134-1136.

47. Gimble, F. S., and R. T. Sauer. 1985. Mutations in bacteriophage lambda repressor that prevent RecA-mediated cleavage. 162:147-154.

48. Glover, S. W., J. Schell, N. Symonds and K. A. Stacey. 1963. The control of host-induced modification by phage P1. Genet. Res. Camb. 4:480-482.

49. Goldberg, I. and J. J. Mekelanos. 1986. Cloning of the Vibrio cholerae recA gene and construction of a Vibrio cholerae recA mutant. J. Bacteriol. 165:715-722.

50. Gonda, D. K. and C. M. Radding. 1983. By searching processively RecA protein pairs DNA molecules that share a limited stretch of homology. Cell 34:647-654.

51. Gottesman, M. M., M. E. Gottesman, S. Gottesman and M. Gellert. 1974. Characterization of bacteriophage lambda reverse as an Escherichia coli phage carrying a unique set of host-derived recombination functions. J. Mol. Biol. 88:471-487.

52. Gudas, L. J. and D. W. Mount. 1977. Identification of the recA (tif) gene product of Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. 74:5280-5284.

53. Gudas, L. J., D. W. Mount and A. B. Pardee. 1975. Model for regulation of Escherichia coli DNA repair functions. Proc. Natl. Acad. Sci. U. S. A. 72:2230-2234.

54. Gudas, L. J., and A. B. Pardee. 1975. DNA synthesis inhibition and the induction of protein X in Escherichia coli J. Mol. Biol. 101: 459-477.

55. Hanawalt, P. C., P. K. Cooper, A. K. Ganesan and C. A. Smith. 1979. DNA repair in bacteria and mammalian cells. Ann. Rev. Biochem. 48:783-836.

56. Hanes, B. D. 1981. An introduction to polyacrylamide gel electrophoresis. p.1 In B. D. Hanes and D. Rickwood (ed.), Gel electrophoresis of proteins. A practical approach. IRL Press, Washington, D. C.

57. Herskowitz, I., and D. Hagen. 1980. The lysis-lysogeny decision of phage lambda: Explicit programming and responsiveness. Ann. Rev. Genet. 14:399-445.

58. Hinkle, N. F., and R. V. Miller. 1979. pMG7-mediated restriction of Pseudomonas aeruginosa phage DNAs is determined by a class II restriction endonuclease. Plasmid 2:387-393.

59. Hochschild, A., N. Irwin and M. Ptashne. 1983. Repressor structure and the mechanism of positive control. Cell 32:319-325.

60. Holloway, B. W. 1966. Mutants of Pseudomonas aeruginosa with reduced recombinational ability. Mutat. Res. 3:452-455.

61. Holloway, B. W., J. B. Egan and M. Monk. 1960. Lysogeny in Pseudomonas aeruginosa. Aust. J. Exp. Biol. 38: 321-330.

62. Holloway, B. W., V. Krishnapillai. 1979. Bacteriophages and bacteriocins, p. 99-132. In P. H. Clarke and M. H. Richmond (ed.), Genetics and biochemistry of Pseudomonas. John Wiley and Sons, New York. N. Y.

63. Holloway, B. W., H. Rossiter, D. Burgess and J. Dodge. Aeruginocin tolerate mutants of Pseudomonas aeruginosa. Genet, Res., Camb. 22:239-253.

64. Holloway, B. W., and P. van de Putte. 1968. Lysogeny and bacterial recombination, p. 175-183. In J. Peacock and R. D. Brock (ed.), Replication and recombination of genetic material. Austrailian Academy of Science, Canberra.

65. Howard-Flanders, P., R. P. Boyce and L. Theriot. 1966. Three loci in Escherichia coli K-12 that control the excision of pyrimidine dimers and certain other mutagen products from DNA. Genetics 53:1119-1136.

66. Hoyt, M. A., D. M. Knight, A. Das, H. I. Miller and H. Echols. 1982. Control of phage lambda development by stability and synthesis of cII protein: Role of the viral cIII nad host hflA, himA and himD genes. Cell 31:565-573.

67. Hulett, F. M. 1984. Cloning and characterization of the Bacillus licheniformis gene coding for alkaline phosphatase. J. Bacteriol. 158:978-982.

68. Inouye, M., and A. B. Pardee. 1970. Changes of membrane proteins and their relation to deoxyribonucleic acid synthesis and cell division. J. Biol. Chem. 245:5813-5819.

69. Itoh, Y., and D. Haas. 1985. Cloning vectors derived from the Pseudomonas plasmid pVS1. Gene 36:27-36.

70. Itoh, Y., L. Soldati, T. Leisinger and D. Haas. 1985. Low, intermediate and high copy number cloning vectors derived from Pseudomonas plasmid pVS1, p. 954. In D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson, A. Hollaender, L. Hager, S. Kaplan and J. Konisky (ed.), Plasmids in bacteria. Plenum Press, New York, N. Y.

71. Itoh, Y., J. M. Watson, D. Haas and T. Leisinger. 1984. Genetic and molecular characterization of the Pseudomonas plasmid pVS1. Plasmid 11:206-220.

72. Jacob, F., and J. Monod. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3:318-356.

73. Jeenes, D. J., L. Soldati, H. Baur, J. M. Watson, A. Mercenier, C. Riemmann, T. Leisinger and D. Haas. 1986. Expression of biosynthetic genes from Pseudomonas aeruginosa and Escherichia coli in the heterologous host. Mol. Gen. Genet. 203:421-429.

74. Johnson, A. D., A. R. Poteete, G. Lauer, R. T. Sauer, K. Ackers and M. Ptashne. 1981. Lambda repressor and Cro - components of an efficient molecular switch. Nature 294:217-223.

75. Kaiser, A. D. 1957. Mutations in a temperate bacteriophage affecting its ability to lysogenize Escherichia coli. Virology 3:42-61.

76. Kaiser, A. D., and F. Jacob. 1957. Recombination between related temperate bacteriophages and the genetic control of immunity and prophage localization. Virology 4:509-521.

77. Karu, A. E., and E. D. Belk. 1982. Induction of E. coli recA protein via recBC and alternate pathways: Quantitation by enzyme-linked immunosorbent assay (ELISA). Mol. Gen. Genet. 185:275-282.

78. Keener, S. L., K. P. McNamee and K. McEntee. 1984. Cloning and characterization of recA genes from Proteus vulgaris, Erwinia carotovora, Shigella flexneri, and Escherichia coli B/r. J. Bacteriol. 160:153-160.

79. Kenyon, C. J., R. Brent, M. Ptashne and G. C. Walker. 1982. Regulation of damage-inducible genes in Escherichia coli. J. Mol. Biol. 160:445-457.

80. Laemlli. U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

81. Leahy, M. C., and C. M. Radding. 1986. Topography of the interaction of RecA protein with single-stranded deoxyoligonucleotides. J. Biol. Chem. 261:6954-6960.

82. Levine, A, A. Bailone and R. Devoret. 1979. Cellular levels of the prophage lambda and 434 repressors. J. Mol. Biol. 131:655-661.

83. Little, J. W. 1984. Autodigestion of lexA and phage lambda repressors. Proc. Natl. Acad. Sci. U. S. A. 81:1375-1379.

84. Little, J. W., S. H. Edmiston, L. Z. Pacelli and D. W. Mount. 1980. Cleavage of the Escherichia coli lexA protein by the recA protease. Proc. Natl. Acad. Sci. U.S.A. 77:3225-3229.

85. Little, J. W., and D. W. Mount. 1982. The SOS regulatory system of Escherichia coli. Cell 29:11-22.

86. Maniatis, T., E. F. Fritsch and J. S. Sambrook. 1982. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory. Cold Spring Harbhemor, NY.

87. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. 3:208-218.

88. McEntee, K. 1977. Protein X is the product of the recA gene of Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. 74:5275-5279.

89. McEntee, K., G. M. Weinstock and I. R. Lehman. 1979. Initiation of general recombination catalyzed in vitro by the recA protein of Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 76:2615-2619.

90. McEntee, K., G. M. Weinstock and I. R. Lehman. 1981. Binding of the recA protein of Escherichia coli to single- and double-stranded DNA. J. Biol. Chem. 256:8835-8844.

91. Menetski, J. P., and S. C. Kowalczykowski. 1985. Interaction of recA protein with single-stranded DNA. Quantitative aspects of binding affinity modulation by nucleotide cofactors. J. Mol. Biol. 181:281-295.

92. Mercer, A. A., and J. S. Loutit. 1979. Transformation and transfection of Pseudomonas aeruginosa: effects of metal ions. J. Bacteriol. 140:37-42.

93. Meselson, M., and J. J. Weigle. 1961. Chromosome breakage accompanying genetic recombination in bacteriophage. Proc. Natl. Sci. U. S. A. 47:857-

94. Miles, C. A., A. Mountain and G. R. K. Sastry. 1986. Cloning and characterization of the recA gene of Agrobacterium tumafaciens CSP. Mol. Gen. Genet. 204:161-165.

95. Miller, J. H. Experiments in molecular genetics. 1972. Cold Spring Harbor Laboratory. Cold Spring Harbor, N. Y.

96. Miller, R. V., and C.-M. C. Ku. 1978. Characterization of Pseudomonsa aeruginosa mutants deficient in the establishment of lysogeny. J. Bacteriol. 134:875-883.

97. Miller. R. V., J. M. Pemberton and A. J. Clark. 1977. Prophage F116: Evidence for an extrachromosomal location in Pseudomonas aeruginosa strain PAO. J. Virol. 22:844-847.

98. Miller, R. V., J. M. Pemberton and K. E. Richards. 1974. F116, D3 and G101: temperate bacteriophages ofPseudomonas aeruginosa. Virology 59:566-569.

99. Moreau, P. L., J. V. Pelico and R. Devoret. 1982. Cleavage of lambda repressor and synthesis of RecA protein induced by transferred F sex factor. Mol. Gen. Genet. 186:170-179.

100. Ogawa, T., and J.-I. Tomizawa. 1967. Abortive lysogenization of bacteriophage lambda b2 and residual immunity of non-lysogenic segregants. J. Mol. Biol. 23:225-245.

101. Ohman, D. E., M. A. West, J. L. Flynn and J. B. Goldberg. 1985. Method for gene replacement in Pseudomonas aeruginosa used in construction of recA mutants: recA independent instability of alginate production. J. Bacteriol. 162:1068-1074.

102. Okii, M., S. Iyobe and S. Mitsuhashi. 1983. Mapping of the gene specifying aminoglycoside 3'-phosphotransferase II on the Pseudomonsa aeruginosa chromosome. J. Bacteriol. 155:643-649.

103. Ossana, N., K. R. Peterson and D. W. Mount. 1986. Genetics of DNA repair in bacteria. Trends in Genetics 2:55-58.

104. Peden, K. W. C. 1983. Revised sequence of the tetracycline resistance gene of pBR322. Gene 22:277-280.

105. Perbal, B. 1984. A practical guide to molecular cloning. John Wiley and Sons. New York, NY.

106. Phyzicky, E. M., and J. W. Roberts. 1980. Kinetics of RecA protein-directed inactivation of repressors of phage lambda and phage P22. J. Mol. Biol. 139:319-328.

107. Ptashne, M. 1967. Isolation of the lambda phage repressor. Proc. Natl. Acad. Sci. U. S. A. 57:306-313.

108. Ptashne, M., K. Backman, M. Z. Humayun, A. Jeffrey, R. Maurer, B. Meyer and R. T. Sauer. 1976. Autoregulation and function of a repressor in bacteriophage lambda. Science 194:156-161.

109. Quillardet, P., P. L. Moreau, H. Ginsburg, D. W. Mount and R. Devoret. 1982. Cell survival, UV reactivation and induction of prophage lambda in Escherichia coli K12 overproducing recA protein. Mol. Gen. Genet. 188:37-43.

110. Register, III, J. C. and J. Griffith. 1986. RecA protein can juxtapose DNA: An activity that may reflect a function in DNA repair. Proc. Natl. Acad. Sci. U. S. A. 83:624-628.

111. Roberts, J. W., and R. Devoret. 1983. Lysogenic induction, p. 123-144. In R. W. Hendrix, J. W. Roberts, F. W. Stahl and R. A. Weisberg (ed.), Lambda II. Cold Spring Harbor Laboratories, Cold Spring Harbor, N. Y.

112. Roberts. J. W. and C. W. Roberts. 1975. Proteolytic cleavage of bacteriophage lambda repressor in induction. Proc. Natl. Acad. Sci. U. S. A. 72:147-151.

113. Roberts, J. W., C. W. Roberts and N. L. Craig. 1978. Escherichia coli recA gene product inactivates phage lambda repressor. Proc. Natl. Acad. Sci. U. S. A. 75:4714-4718.

114. Roberts, J. W., C. W. Roberts and D. W. Mount. 1977. Inactivation and proteolytic cleavage of phage lambda repressor in vitro in an ATP-dependent reaction. Proc. Natl. Acad. Sci. U. S. A. 74:2283-2287.

115. Rolfe, B., and B. W. Holloway. 1966. Alterations in host specificity of bacterial deoxyribonucleic acid after an increase in growth temperature of Pseudomonas aeruginosa. J. Bacteriol. 92:43-48.

116. Rupp, W. D., and P. Howard-Flanders. 1968. Discontinuities in the DNA synthesized in an excision-deficient strain of Escherichia coli following ultraviolet irradiation. J. Mol. Biol. 31:291-304.

117. Rupp, W. D., C. E. Wilde and D. L. Reno. 1971. Exchanges between DNA strands in ultraviolet-irradiated Escherichia coli. J. Mol. Biol. 61:25-44.

118. Ruvkun, G. B., and F. M. Ausubel. 1981. A general method for site-directed mutagenesis in prokaryotes. Nature 289: 85-88.

119. Sancar, A. and W. D. Rupp. 1979. Physical map of the recA gene. Proc. Natl. Acad. Sci. U. S. A. 76:3144-3148.

120. Sancar, A., C. Stachelek, W. Konigsberg and W. D. Rupp. 1980. Sequences of the recA gene and protein. Proc. Natl. Acad. Sci. 77:2611-2615.

121. Sano, Y., and M. Kageyama. 1984. Genetic determinant of pyocin AP41 in the Pseudomonas aeruginosa chromosome. J. Bacteriol. 158:562-570.

122. Schoemaker, J. M. , R. C. Gayda and A. Markovitz. 1984. Regulation of cell division in Escherichia coli : SOS induction and cellular location of the SulA protein, a key to lon-associated filamentation and cell death. J. Bacteriol. 158:551-561.

123. Scurlock, T. R., and R. V. Miller. 1979. PaeExoIX: A unique deoxyribonuclease from Pseudomonas aeruginosa active in the presence of EDTA. Nuc. Acids Res. 7:167-177.

124. Sedgwick, S. G. 1975. Inducible error-prone repair in Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. 72:2753-2757.

125. Sedgwick, S. G., and P. A. Goodwin. 1985. Differences in mutagenic and recombinational DNA repair in Enterobacteria. Proc. Natl. Acad. Sci. U. S. A. 82:4172-4176.

126. Sedgwick, S. G. and G. T. Yarronton. 1982. Cloned truncated recA genes in Escherichia coli. I. Effect on radiosensitivity and recA^{+} dependent processes. Mol. Gen. Genet. 185:93-98.

127. Setlow, R. B., and W. L. Carrier. 1964. The disappearance of thymine dimers from DNA: An error-correcting mechanism. Proc. Natl. Acad. Sci. U. S. A. 51:226-231.

128. Shibata,T., C. DasGupta, R. P. Cunningham and C. M. Radding. 1979. Purified Escherichia coli recA

protein catalyzes homologous pairing of superhelical DNA and single-stranded fragments. Proc. Natl. Acad. Sci. U. S. A. 76:1638-1642.

129. Shilhavy,T. J., M. L. Berman and L. W. Enquist. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

130. Shinomyia, T., M. Ohsumi and M. Kageyama. 1975. Defective pyocin particles produced by some mutants strains of Pseudomonas aeruginosa. J. Bacteriol. 124:1508-1521.

131. Stent, G. S., and R. Calendar. 1971. Molecular genetics. An introductory narrative. W. H. Freeman and Co. San Francisco, CA.

132. Tessman, E. S., and P. K. Peterson. 1985. Isolation of protease-proficient, recombinase-deficient recA mutants of Escherichia coli K-12. J. Bacteriol. 163:688-695.

133. Theile, M., S. Scherneck and E. Geissler. 1981. Influence of superinfection on the photoreversible phase of UV-induced lysogenic bacteria. 1981. Mol. Gen. Genet. 181:273-278.

134. Thomas, P. S. 1980. RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U. S. A. 77:5201-5205.

135. Tsang, S. S., K. Muniyappa, E. Azhderian, D. K. Gonda, C. M. Radding, J. Flory and J. W. Chase. 1985. Intermediates in homologous pairing promoted by recA protein. Isolation and characterization of active presynaptic complexes. J. Mol. Biol. 185:295-309.

136. van de Putte, P., and B. W. Holloway. A thermosensitive recombination deficient mutant of Pseudomonas aeruginosa. Mutat. Res. 6:195-203.

137. Volkert, M. R., and M. A. Hartke. 1984. Suppression of Escherichia coli recF mutations by recA-linkedsrfA mutations. J. Bacteriol. 157:498-506.

138. Volkert, M. R., L. J. Margossian and A. J. Clark. 1984. Two-component suppression of recF143 by

recA441 in Escherichia coli K-12. J. Bacteriol. 160:702-705.

139. Wacker, A. 1963. Molecular mechanisms of radiation effects. Direct correlation between cell survival and amount of thymine dimers detected. Prog. Nuc. Acids Res. 1:369-399.

140. Wahl,G. M., M. Stern and G. R. Stark. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. U. S. A. 76: 3683-3687.

141. Walker, G. C. 1984. Mutagenesis and inducible responses to deoxyribonucleic acid damage in Escherichia coli. Microbiol. Rev. 48:60-93.

142. Wang, W-. B. and E. S. Tessman. 1985. Evidence that the recA441 (tif-1) mutant of Escherichia coli K-12 contains a thermosensitive intragenic supressor of RecA constitutive protease activity. J. Bacteriol. 163:401-403.

143. Weigle, J. 1953. Induction of mutations in a bacterial virus. Proc. Natl. Acad. Sci. U. S. A. 39:628-636.

144. Weigle, J. J. 1966. Assembly of phage lambda in vitro. Proc. Natl. Acad. Sci. U. S. A. 55:1462-1466.

145. Weigle, J. J., and M. Delbruck. 1951. Mutual exclusion between an infecting phage and a carried phage. J. Bacteriol. 62:301-318.

146. Weinstock, G. M., K. McEntee and I. R. Lehman. 1979. ATP-dependent renaturation of DNA catalyzed by the recA protein of Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. 76:126-130.

147. Weinstock, G. M., K. McEntee and I. R. Lehman. 1981. Hydrolysis of nucleoside triphosphates catalyzed by the recA protein of Escherichia coli J. Biol. Chem. 256:8829-8834.

148. Weisemann, J. M., C. Funk and G. M. Weinstock. 1984. Measurement of in vivo expression of the recA gene of escherichia coli by using lacZ fusions. J. Bacteriol. 160:112-121.

149. West, S. C., E. Cassuto and P. Howard-Flanders. 1981. Heteroduplex formation by recA protein. Proc. Natl. Acad. Sci. U. S. A. 78:6149-6153.

150. West, S. C., E. Cassuto, J. Mursalim and P. Howard-Flanders. 1980. Recognition of duplex DNA containing single-stranded regions by recA protein. Proc. Natl. Acad. Sci. U. S. A. 77:2569-2573.

151. Witkin, E. M. 1967. The radiation sensitivity of Escherichia coli B: A hypothesis relating filament formation and prophage induction. Proc. Natl. Acad. Sci. U. S. A. 57:1275-1279.

152. Witkin, E. M. 1974. Thermal enhancement of ultraviolet mutability in a tif-1, uvrA derivative of Escherichia coli B/r: Evidence that ultraviolet mutagenesis depends upon an inducible function. Proc. Natl. Acad. Sci. U. S. A. 71:1930-1934.

153. Witkin, E. M. 1976. Ultraviolet mutagenesis and inducible DNA repair in Escherichia coli. Bacteriol. Rev. 40:869-907.

APPROVAL SHEET

The dissertation submitted by Tyler Alan Kokjohn has been read and approved by the following committee:

Dr. Robert V. Miller, Director
Professor, Biochemistry and Biophysics
Loyola University of Chicago

Dr. Stephen K. Farrand
Associate Professor, Plant Pathology
University of Illinois, Urbana

Dr. Allen Frankfater
Associate Professor, Biochemistry and Biophysics
Loyola University of Chicago

Dr. Philip Matsumura
Associate Professor, Biological Sciences
University of Illinois, Chicago

Dr. Robert E. Malone
Associate Professor, Biology
University of Iowa

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

15 June, 1987 — Robert V. Miller
Date — Director's Signature